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facts that no methoxyl or *N*-methyl group has been shown to occur in proteins, no other compound containing a methylthiol or similar group has been isolated and lastly that the amino-acids other than methionine do not liberate volatile iodide when treated with hydriodic acid. Such determinations together with those of sulphydryl and disulphide sulphur have enabled Baernstein [1932, 2] to account for the total sulphur in a large number of proteins, though keratins were not studied. In many instances the methionine content is high and with caseinogen methionine-sulphur accounts for 84 % of the total sulphur. The validity of the assumption that methionine is the sole source of volatile iodide may be questionable, but such determinations are not without significance in that they appear to give at least an upper limit for methionine content.

A series of determinations of methionine on various types of wool was made, a detailed account of the modifications found necessary being given below.

Table I.

	Quality*	Total sulphur on dry wt. %	Methionine on dry wt. %	% of total sulphur as methionine-sulphur
Welsh Mountain S. 64	58s	3·97†	0·44	2·4
Welsh Mountain Kemp	Very coarse	3·29	0·64	4·1
Romney Corriedale	36s	2·96†	0·66	4·8
Camel Hair	80s	3·37	0·67	4·3
Scotch Blackface (fine)	56s	3·73	0·57	3·2
Scotch Blackface (coarse)	Very coarse	3·10	0·51	3·4
Alpaca	60s	3·68	0·54	3·2
Mohair	36s	3·20	0·63	4·3
Lincoln	36s	3·46	0·50	3·0
Western Australian	70s	3·73	0·55	3·2
New Zealand	36s	3·40	0·50	3·2

* The qualities of the samples were determined by comparison with standard wools, and the author is indebted to Miss A. L. Walker for these determinations. Qualities of 80s, 70s, 60s, 56s and 36s correspond to diameters of 1·92, 2·07, 2·24, 2·83 and $3{\cdot}90 \times 10^{-3}$ cm. respectively.

† Indicates sulphur by the Carius method, others by the Benedict-Denis.

Experimental.

Preparation of samples. The wool samples were removed from the fleece in locks, which were opened out and degreased by washing in three baths of benzene at 60°. Small particles of foreign matter were removed by hand-sorting and the wools given a final treatment with benzene. The samples were dried at room temperature and well washed in six changes of distilled water, air-dried, and allowed to attain a uniform moisture content. Samples for methionine, sulphur and moisture content determinations were weighed off. The dry weights of individual samples were obtained by reference to determinations of moisture content made on samples taken at the beginning and end of the sampling. The moisture determination on the reference sample was carried out by passing a stream of dry air through the wool contained in a special bottle, maintained at 106° in an electrically heated oven, the procedure being fully described in an earlier paper [Barritt and King, 1926].

Determination of methionine. The technique employed was substantially that due to Baernstein [1932, 1] though with wool minor modifications were necessary.

Using a single washing-tube containing cadmium sulphate and a suspension of red phosphorus, it was found that a little hydrogen sulphide escaped and passed over into the alcoholic silver nitrate. This difficulty probably arises because of the very high cystine content of the wool-keratin and was overcome

by inserting a small tube containing glass wool or paper pulp moistened with cadmium sulphate solution between the washing-tube and the first silver nitrate tube. As a further check on the efficiency of the removal of hydrogen sulphide, a cadmium sulphate test-paper was inserted immediately before the first silver nitrate tube; if any signs of yellowing of the paper occurred, the experiment was abandoned.

In order to prevent the boiling hydriodic acid sucking up the side-tube and into the sulphuric acid wash-bottle, a bulb 2–3 cm. in diameter was blown on the side-tube; it was found to be quite effective. The duration of the run was 7–8 hours, this being sufficient in the case of wool-keratin.

Alcoholic silver nitrate (10 ml. of the order of $N/10$) was distributed between the two absorption-tubes. At the end of the run the tubes were washed out into a small beaker and the liquid evaporated on the steam-bath to about 10 ml. and made up to 50 ml. The contents of the flask were filtered through a dry paper and 20 ml. aliquots titrated with potassium thiocyanate (order of $N/50$), 0·5 ml. ferric alum indicator being added. The amount of volatile iodide was readily obtained from these data.

The blank on the apparatus was calculated from the results of eight determinations on each wool, four using 0·5 g. and four using 1 g. samples, these being taken alternately. A typical run is shown in detail as follows:

Welsh Mountain Wool. Sheep S. 64.

No. of exp. ...	1	2	3	4	5	6	7	8
Wt. of wool taken (g.)	0·5867	1·0658	0·5154	0·9535	0·5342	0·9467	0·4770	1·0640
ml. of 0·01933 KCNS equivalent to AgI	1·40	1·91	1·28	1·97	1·21	1·76	1·15	1·99

Moisture: 0·9698 g. of conditioned wool on drying out lost 0·1194 g.

Combining the above results, the blank on the run corresponds to 0·55 ml. and the methionine content of the wool to 1·35 ml. KCNS per g., giving 0·44 % methionine on the dry weight of the wool.

The value of the blank so obtained was found to agree well with values obtained when no wool was added. Although the blank is high in relation to the amount of KCNS corresponding to the methionine it is felt that the method employed is satisfactory in determining with reasonable accuracy the methyl iodide produced from wool-keratin.

Determination of sulphur content of wool. This was determined either by the Carius method using samples of 0·5 g. [Barritt and King, 1926] or by the method of Benedict [1909] and Denis [1910].

Using the latter method a sample of 0·8 to 1 g. was hydrolysed for 2 to 4 hours with 20 ml. 6N hydrochloric acid, the hydrolysate being made up to 50 ml. Aliquots of 20 ml. were taken and the determination then proceeded with as described by Rimington [1930]. This method is somewhat inaccurate owing to the fact that on hydrolysis, particularly in the early stages, small quantities of hydrogen sulphide are liberated. It is hoped to deal more fully with the determination of total sulphur in keratins in a separate paper, but in view of the work of Gortner [1922], which shows that no appreciable decomposition of cystine occurs during the time necessary for a protein hydrolysis, it is suggested that this liberated hydrogen sulphide arises from the methionine or some unknown sulphur compound of wool-keratin.

Discussion.

The results given in the above table for a fairly representative series of wools do not justify any rigorous correlation between the methionine content and the type of wool, as was possible with the total sulphur in wools, where it was shown [Barritt and King, 1926] that total sulphur is higher in the finer wools grown under similar conditions. The sample of camel hair is of interest in that only 94 % of its total sulphur could be accounted for as cystine-sulphur [Rimington, 1929, 2] but its methionine content is not abnormally high.

The methionine content of the wools examined lies between 0·44 and 0·67 % of the dry weight, the methionine-sulphur varying from 2·4 to 4·8 % of the total sulphur. This quantity of methionine-sulphur accounts for about 3–4 % of the total sulphur in wool and does not seriously affect the previous conclusion that sulphur in wool occurs almost wholly in the form of cystine, though the possibility of undiscovered sulphur-containing amino-acids must not be overlooked. A possible explanation for the somewhat high values recorded for cystine-sulphur in keratin using the Folin-Marenzi technique is afforded by the work of Butz and du Vigneaud [1932] on the hydrolysis of methionine. On hydrolysis with acids, especially sulphuric acid, methionine gives homocystine, the next higher homologue of cystine, which is reactive to the Folin-Marenzi reagent; thus it appears possible that some methionine-sulphur may have been returned as cystine-sulphur using the colorimetric method of Folin-Marenzi.

The actual synthesis of wool-protein by the sheep is somewhat outside the scope of the present work, but it is interesting to note that the form in which the sulphur is obtained has been the subject of much speculation. Many calculations have been made of the necessary cystine content of herbage [Rimington and Bekker, 1932; Henrici, 1932; King, 1933; Woodman and Evans, 1932; Robertson, 1928] based on the assumption that cystine as such is essential for ultimate wool synthesis. These calculated values vary widely, some falling within, others outside the values for cystine in grasses as determined by Aitken [1930] and Evans [1931].

In determining cystine values obtained on a grass hydrolysate, the probability of loss of cystine due to interaction with carbohydrate material must be considered, and recently Lugg [1933] has shown that considerable loss of cystine occurs when it is hydrolysed by hydrochloric acid in the presence of sucrose and glycine. Preliminary determinations (by the author) of cystine in straw and potatoes show very high values, using the Folin-Marenzi technique, but only small amounts with the Sullivan reagent, illustrating the difficulties which occur when cystine is directly determined in complex materials.

Assuming a deficiency of cystine in the herbage, Rimington and Bekker [1932] have suggested that inorganic sulphur is elaborated by micro-organisms of the intestinal tract into bacterial protein which is then absorbed and is available for cystine synthesis, some support being given to this hypothesis by the work of Voltz [1920].

The total organic sulphur in herbage [Aitken, 1930; Evans, 1931] is however well above the sulphur requirements of wool production, but its actual distribution is as yet undetermined, though the work of Miller and Chibnall [1932] suggests the presence of methionine. In view of the possible replacement of cystine by methionine in growth experiments [Weichselbaum *et al.*, 1932; Jackson and Block, 1932], the suggestion of Jackson and Block, that cystine and methionine form a freely interconvertible system of which only one member is indispensable to a limited degree, and that an adequate supplement

of the other may suffice to provide a common metabolite of the two, is of especial interest in a study of wool production, though their experimental data do not eliminate the possibility of alternative explanations.

SUMMARY.

1. The methionine content of various wools has been determined. It is shown that the amount present does not substantially affect previous work on the relation between total and cystine-sulphur in wool, which indicated that substantially all the sulphur in wool could be accounted for as cystine.

2. The suggestion is made that methionine occurs widely in feeding stuffs and grasses and may play an important rôle in the ultimate synthesis of wool- and hair-proteins.

The author wishes to express his thanks to the Council of the Wool Industries Research Association for permission to publish this account, and to Prof. A. T. King, formerly Head of the Chemistry Department, for the interest he has taken in the work. Throughout the course of the investigation, the author's assistant Mr F. F. Elsworth, has given valuable help.

REFERENCES.

Aitken (1930). *Biochem. J.* **24**, 250.
Baernstein (1932, 1). *J. Biol. Chem.* **97**, 663.
—— (1932, 2). *J. Biol. Chem.* **97**, 669.
Barger and Coyne (1928). *Biochem. J.* **22**, 1417.
Barritt (1927). *J. Soc. Chem. Ind.* **46**, 338.
—— and King (1926). *J. Text. Inst.* **17**, 386.
—— and Rimington (1931). *Biochem. J.* **25**, 1072.
Benedict (1909). *J. Biol. Chem.* **6**, 363.
Butz and du Vigneaud (1932). *J. Biol. Chem.* **99**, 135.
Dakin (1920). *J. Biol. Chem.* **44**, 499.
Denis (1910). *J. Biol. Chem.* **8**, 401.
Evans (1931). *J. Agric. Sci.* **21**, 806.
Folin and Marenzi (1929). *J. Biol. Chem.* **83**, 103.
Gortner (1922). *J. Amer. Chem. Soc.* **24**, 341.
Henrici (1932). Farming in South Africa, 245.
Jackson and Block (1932). *J. Biol. Chem.* **98**, 465.
Jones and Gersdorff (1933). *J. Biol. Chem.* **101**, 657.
King (1933). *Trans. Faraday Soc.* **29**, 258.
Kirpal and Buhn (1915). *Monats. Chem.* **36**, 853.
Lugg (1933). *Biochem. J.* **27**, 1022.
Marston (1928). *Commonwealth of Australia, C.S.I.R. Bull.* 38.
Miller and Chibnall (1932). *Biochem. J.* **26**, 392.
Mueller (1921). *Proc. Soc. Exp. Biol. Med.* **19**, 161.
—— (1923). *J. Biol. Chem.* **56**, 157.
Pirie (1932). *Biochem. J.* **26**, 1270.
Pollack and Spitzer (1922). *Monats. Chem.* **43**, 113.
Rimington (1929, 1). *Biochem. J.* **23**, 41.
—— (1929, 2). *Biochem. J.* **23**, 726.
—— (1930). *J. Soc. Chem. Ind.* **49**, 139.
—— and Bekker (1932). *Nature*, **129**, 687.
Robertson (1928). *Commonwealth of Australia, C.S.I.R. Bull.* 39.
Sullivan (1926). *U.S. Public Health Rep.* **41**, 1030.
Voltz (1920). *Biochem. Z.* **102**, 151.
Weichselbaum, Weichselbaum and Stewart (1932). *Nature*, **129**, 795.
Woodman and Evans (1932). *Nature*, **130**, 1001.

II. INOSITOL METABOLISM IN THE MAMMALIAN HEART.

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The significance of the occurrence of inositol in animal tissues is still unexplained, and it is a matter of dispute whether inositol is metabolised by animals. Rosenberger [1910] asserted that the inositol content of muscle was increased during autolysis but gave no figures. Needham [1923] compared the inositol contents of different tissues of a freshly killed rabbit and of one killed two days before: he remarks "It is certainly interesting that the muscle from the freshly killed rabbit contained 50 % less inositol than that from the rabbit which had been dead two days. It recalls the view of Rosenberger that tissues contain an 'Inositogen,' though his conclusions were drawn from the comparison of fresh rabbit muscle and commercial beef; tissues perhaps hardly comparable."

Boyland [1928], on the other hand, records three experiments with pig's heart and as a result suggests that inositol decreases during survival changes in muscle and is a precursor of lactic acid. For a review of the literature see Needham [1926].

Experimental.

Work on inositol has been hampered by lack of a convenient method for its estimation. Needham [1923] made use of acetone for extraction of the tissues, and precipitated the inositol with basic lead acetate: the inositol was then thrown down with strong alcohol and the carbon in the precipitate was determined. Boyland [1928] used a modification of Needham's method, the modification consisting in a direct weighing of the precipitate in place of a micro-estimation of carbon. Boyland remarks that "The method is probably not very specific, but a more reliable method could not be found." Complete extraction of even minced tissue is difficult unless large quantities of solvent are used, and then it is not easy to make the process quantitative: in addition, in the present work it was desirable to use a method by which cell enzymes could be destroyed or inactivated as soon as possible after removal of the tissue from the body. It was decided to take advantage of the stability of inositol to hot alkali. Rosenberger [1910] made use of boiling KOH solution for the estimation of inositol, but his drastic treatment has been criticised by Momose [1916]. The principle of the method employed in the present work is as follows. The tissue (30–40 g.) is disintegrated by heating with an equal volume of 10 % aqueous KOH: after neutralisation and cooling, a large amount of impurity is removed with normal lead acetate: the inositol is precipitated by basic lead acetate, and after being freed from the lead compound is precipitated by baryta in alcoholic solution. After decomposition of the barium precipitate the inositol is dried, recrystallised and weighed. The fresh heart-muscle of dogs was used throughout. The details are best described in the form of a typical experiment.

The animal is killed by severing the carotid arteries; the chest is immediately opened and the heart removed[1]. Excess of fat is removed with a few strokes of the knife, the heart is divided roughly into two, one-half is immediately weighed and then cut up over a flask containing 10 % KOH at 100°; when all the pieces have been dropped into the potash a condenser is fitted and boiling is continued for the required period. The amount of KOH solution should be about 1 ml. for each gram of tissue. Meanwhile the other half of the heart is allowed to undergo survival changes, then the muscle is cut up into potash and the procedure is the same as for the first half. After the boiling with alkali is completed the fluid is made acid to litmus with glacial acetic acid, cooled to about 50°, and a rapid current of hydrogen is passed through for an hour to remove hydrogen sulphide liberated by the action of the potash on sulphur compounds in the muscle. Half the volume of water is added, and after standing for a day in the cold chamber the fluid will filter clear. To the filtrate is added a saturated solution of normal lead acetate, 0·2 ml. per g. of muscle, and a day is allowed for complete precipitation.

After centrifuging (the precipitate being discarded without washing) ammonia is added to the clear fluid until further additions give no precipitate. The precipitate is very bulky, but may be collected into two centrifuge-tubes of 25 ml. capacity if a very high speed machine such as the "Leune," made by Messrs Hearson, is used. To the clear liquid lead acetate (0·2 ml. per g. of tissue) is added, followed by ammonia. The precipitate is collected in the same cups as the first. A further precipitation, using half the quantity of lead, is performed. The combined precipitate is twice washed very carefully with 75 % ethyl alcohol, and the cups with the precipitate are then dried *in vacuo* over sulphuric acid. The precipitate is then weighed and collected in one centrifuge-cup; it is ground up with 2*N* sulphuric acid and about 5 ml. of water until the mixture is acid to litmus. About 2 ml. of acid are required for each g. of the lead compound. The mixture is centrifuged and the liquid is measured and poured into a 100 ml. flask. The precipitate is stirred up with 5 ml. of water, and sufficient acid (about 0·3 ml.) is added to make the reaction acid. The process is repeated. To the combined extracts in the flask 3 volumes of strong alcohol are added. Precipitation takes several days. The fluid is centrifuged, and the precipitate is discarded without washing. An excess of 10 % baryta in methyl alcohol is added, and after 10 minutes the liquid is centrifuged and the precipitate washed with 75 % alcohol, and suspended in 100 ml. of 75 % alcohol in a tall cylinder. Care must be taken to obtain a uniform suspension, and this is best effected by first stirring up the precipitate with very small amounts of alcohol. The barium-inositol compound is decomposed by passing in a stream of CO_2 at 40° for 2 hours. Two days are allowed for precipitation of carbonate, the fluid is centrifuged and concentrated to dryness under reduced pressure. The residue is washed with dry methyl alcohol, taken up in 5 ml. water and allowed to evaporate slowly over sulphuric acid. Usually the inositol crystallises but occasionally is left as a glass. Recrystallisation is effected by dissolving the solid in 2·5 ml. water and adding 20 ml. strong alcohol. After 2 weeks the crystals are washed with methyl alcohol, dried and weighed. The melting-points of different preparations made in this way varied between 211 and 215°. Two preparations taken at random gave the following analytical figures (micro-analyses Schoeller) C 40·36, H 6·79 %; C 40·44, H 6·75 %; $C_6H_{12}O_6$ requires C 40·00, H 6·66 %.

[1] Dr T. H. B. Bedford removed all the hearts, and I am grateful for the rapidity and skill which he showed in all these experiments.

To determine whether survival changes in heart muscle affected the inositol content, one half heart was immediately treated with boiling potash, while the other was kept in an atmosphere of nitrogen for 90 minutes at 37°. Each was boiled with potash for 30 minutes. The following four experiments show that the inositol content is increased on incubation of the tissue.

Muscle killed immediately mg. inositol per 100 g.	Muscle after survival changes mg. inositol per 100 g.
22·3	29·5
10·0	40·0
30·2	61·6
10·0	23·7

In the following series the time of boiling was increased, to determine whether inositol was being set free from some combination by the action of the alkali.

	Muscle killed immediately mg. inositol per 100 g.	Muscle after survival changes mg. inositol per 100 g.
Boiled for 1 hour	7·8	8·3
	54·1	54·0
	9·2	79·3
	40·5	45·0
Boiled for 2 hours	43·9	68·3
	64·9	74·5
	120·0	128·0
	122·0	166·0
Boiled for 5 hours	79·0	114·0
	112·3	122·0
	147·0	185·0

Two facts emerge from this series: the amount of inositol increases with the time of action of the alkali, and the survival changes still cause more inositol to be formed than in the corresponding half heart killed immediately. To decide whether 5 hours' boiling with 10 % KOH is sufficient to liberate the whole of the "combined" inositol, hearts were divided into two, and one half was boiled for 5 hours, the other for 12; each half was thrown into potash immediately after weighing.

5 hours' boiling mg. inositol per 100 g.	12 hours' boiling mg. inositol per 100 g.
108·3	94·0
40·2	34·4
61·7	61·0
45·7	59·5

It is evident that the maximum liberation of inositol by the potash is effected in 5 hours, within the limits of experimental error of the method, necessarily large. Four experiments were performed to estimate this error, the heart being divided into two equal pieces and the inositol estimated after boiling for 5 hours.

mg. inositol per 100 g.		Percentage error	Average error
108·8	101·2	7·5	
119·0	106·2	12·1	
68·7	53·0	29·6	±13 %
78·9	75·3	4·8	

That the maximum yield of inositol from heart muscle is found after survival changes have taken place is confirmed by the following series of experiments, in which the hearts were divided into three approximately equal parts, A—killed

at once and boiled 30 minutes, B—killed at once and boiled 5 hours, C—incubated 90 minutes in nitrogen at 37° and boiled 5 hours.

Muscle killed before survival changes		After survival changes
A. Boiled ½ hour	B. Boiled 5 hours	C. Boiled 5 hours
mg. inositol per 100 g. tissue.		
59·7	83·0	102·0
12·8	18·6	36·8
49·5	71·0	102·0
83·0	112·0	141·0

In individual hearts, the value of A is that of the "free" with a small amount of the "combined" inositol, for it must be assumed that boiling for even half an hour with potash liberates some inositol from combination: the difference between A and B gives the value for the "combined" inositol (too low, for the reason already mentioned), and the difference between B and C is the amount of inositol produced during survival changes in the muscle; this will be referred to as the "new-formed" inositol. All the above experiments were carried out with a minimum of cell destruction. To obtain further light on the mechanism of production of the "new-formed" inositol, it was desirable to determine whether moderate cell disintegration, as in mincing, interferes appreciably with the production of the "new-formed" inositol. The hearts were minced and divided into two nearly equal parts. One, after weighing, was placed at once in twice the usual volume of boiling potash (10 % in 0·9 % sodium chloride) and the other was incubated in 0·9 % saline (1 ml. for each g. tissue) for 90 minutes at 37° in an atmosphere of nitrogen. At the end of this time 20 % potash in amount equal to the original volume of saline was added. Boiling was for 5 hours in each case.

Muscle killed immediately	After survival changes
mg. inositol per 100 g. tissue.	
60·0	69·5
78·6	96·6
87·6	95·0
78·4	97·0

It is evident that new formation of inositol has taken place. The possible effect of phosphate buffers of different p_H was then tried. The hearts were minced and divided as nearly as possible into three equal parts. Column A refers to the tissue which was placed at once in 10 % potash. In column B are the figures for the tissue incubated for 1½ hours in buffer p_H 5, in column C those for p_H 8·0. All were boiled for 5 hours with the same volume and concentration of alkali.

A	B	C
mg. inositol per 100 g. tissue.		
72·0	66·0	66·0
44·0	73·0	65·7
58·7	61·6	71·5
62·9	92·0	87·0

In three of the experiments the yield of inositol was increased on incubation of the muscle, but there was no significant difference between the acid and alkaline media.

In the following series of experiments a known amount of inositol was added to one half heart, and the whole extraction process was carried through to estimate how much of the substance is lost in the method of extraction and to obtain approximate figures for the free, combined, and "new-formed" inositol in the dog's heart. The average recovery was 73·2 %.

mg. inositol added	mg. inositol in each half heart		Difference	Percentage recovered
71·9	28·4	80·0	51·6	71·8
76·0	13·6	65·5	51·9	68·1
85·0	16·2	78·6	62·4	73·5
79·2	15·5	78·8	63·2	79·3

Discussion.

Though the experimental error of the method is considerable, it has the advantage of certainty that inositol is being estimated, and it seems safe to conclude that in the heart muscle of the dog the inositol is of three kinds. Firstly, that lying free in the muscle: it is difficult to say what proportion this bears to the whole, for survival changes were occurring between the death of the animal and the killing of the muscle cells in the boiling potash, and there is also the hydrolytic action of the alkali to be considered during the 30 minutes' boiling. It may be that there is no free inositol in muscle, and if steps were taken to reduce survival changes to a minimum, a very small value might be obtained. Secondly, the combined inositol, that which is liberated completely after 5 hours' boiling with potash: the source of this is unknown. Thirdly, the "new-formed" inositol, that which appears during survival changes in the muscle on incubation in an atmosphere of nitrogen: the possibilities are that it is synthesised, or that it is liberated by enzymic action from some combination which is not split by boiling alkali. These problems must be left for further consideration.

Summary.

1. A method is described for the estimation of inositol in muscle.
2. Survival changes in the heart muscle of the dog are accompanied by an increase in the inositol content.
3. Evidence is brought forward for the existence of free, combined and "new-formed" inositol in the heart muscle.

It is a pleasure to thank Prof. H. S. Raper for his interest and advice throughout the work. Part of the expense was defrayed by the Government Grant Committee of the Royal Society.

References.

Boyland (1928). *Biochem. J.* **22**, 262.
Momose (1916). *Biochem. J.* **10**, 120.
Needham (1923). *Biochem. J.* **17**, 422.
—— (1926). *Ergebn. Physiol.* **25**, 1.
Rosenberger (1910). *Z. physiol. Chem.* **64**, 341

III. CHEMICAL PROPERTIES OF PUBERULIC ACID, $C_8H_6O_6$, AND A YELLOW ACID, $C_8H_4O_6$.

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The two acids formed from glucose by *Penicillium puberulum* Bainier and *P. aurantio-virens* Biourge were first isolated and investigated by Birkinshaw and Raistrick [1932]. Further investigations are recorded below.

Puberulic acid $C_8H_6O_6$.

Improved isolation of puberulic acid. The starting material, which was kindly supplied by Prof. Raistrick, consisted of a mixture of puberulic acid and the yellow acid $C_8H_4O_6$. This mixture had been obtained by crystallisation from methyl alcohol of the ethereal extract from the acidified nickel salts, as described by Birkinshaw and Raistrick [1932], and their method was followed in the preparation of well crystallised diacetylpuberulic acid. Dilution of the acetylation mixture with glacial acetic acid has proved to be useful in order to minimise charring. Diacetylpuberulic acid in alcoholic solution gives with $FeCl_3$ first a reddish brown and then a green colour. Brief hydrolysis with hot aqueous ammonia (a much milder method than the boiling with *N* sodium hydroxide used by Birkinshaw and Raistrick [1932]) yields analytically pure puberulic acid which may be recrystallised from water, and it is then obtained as small, practically colourless prisms. One can thus dispense with the final purification by means of high vacuum sublimation, which is attended by such losses as to give yields of only 10–15 %.

Diacetylpuberulic acid, triturated with a little water, is mixed with a small amount of concentrated ammonia solution and heated for about 10 mins. on the water-bath in a flask loosely corked in order to avoid too rapid loss of ammonia. After cooling and acidification by hydrochloric acid, a fine crystalline powder separates which should not show any needles of the unchanged diacetyl compound under the microscope. Yield 90 %. (Found: C, 48·40; H, 3·33 %. Calc.: C, 48·47; H, 3·05 %.) Recrystallisation from water must be carried out quickly; the substance is not very soluble in hot water; M.P. 300–305° (decomp.). (Found for recrystallised product: C, 48·2; H, 3·16 %.)

Decarboxylation of puberulic acid. Careful sublimation of puberulic acid at 290–310° under atmospheric pressure yields a crystalline product which is obtained in uniform, thin, glittering leaflets when the sublimation is repeated several times. This sublimed product may be recrystallised from absolute alcohol, M.P. 233–235°; reddish brown colour reaction with $FeCl_3$. (Found: C, 54·34; H, 4·37 %. $C_7H_6O_4$ requires: C, 54·53; H, 3·93 %.)

The only benzenemonocarboxylic acid agreeing in formula and M.P. with decarboxylated puberulic acid is 3:5-dihydroxybenzoic or α-resorcylic acid (M.P. 233°; no colour reaction with $FeCl_3$). Since the M.P. of the mixture showed a depression of 32°, these two acids are not identical.

Further evidence against puberulic acid being a benzenedicarboxylic acid. Since puberulic acid gives a distinct fluorescence when fused with resorcinol and zinc chloride (at 130–140° in an oil-bath for 10 mins.), the possibility that it might be a benzenedicarboxylic acid was again considered, although Birkinshaw and Raistrick's negative findings seemed conclusive. As a series of experiments with various aliphatic and aromatic alcohols, mono- and di-carboxylic acids showed, and as Damm and Schreiner [1882] had stated, this fluorescence does not afford a definite proof of the presence of two carboxyl groups. It was possible, however, that we were not dealing with quite the same compound as Birkinshaw and Raistrick, in view of the different conditions of hydrolysis. We therefore compared puberulic acid—prepared by the new ammonia method—with 2:4-dihydroxybenzene-1:3-dicarboxylic acid and with 4:6-dihydroxybenzene-1:3-dicarboxylic acid. We are indebted to Prof. Raistrick for specimens of these acids, the only ones deserving consideration; they are distinct from puberulic acid, which cannot therefore be a benzenedicarboxylic acid.

On the possibility of puberulic acid being a phenolic monocarboxylic acid. The alkaline hydrolysis of tetramethylpuberulic acid yields interesting information.

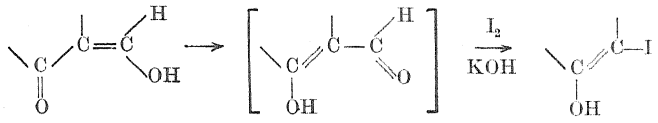
Hydrolysis at room temperature. A solution of tetramethylpuberulic acid in a measured amount of *N* NaOH was allowed to stand for 5–6 mins. at 21° and was then neutralised with *N* H_2SO_4. Long, pale yellow needles crystallised out, and after recrystallisation from water had M.P. 204° and gave no colour reaction with $FeCl_3$. They consisted of a trimethylpuberulic acid. (Found: C, 54·81; H, 5·25 %. $C_{11}H_{12}O_6$ requires: C, 54·98; H, 5·04 %.)

Hot hydrolysis, by boiling tetramethylpuberulic acid with a measured excess of *N* NaOH for 1 hour in an atmosphere of N, showed the consumption of only one equivalent of alkali on back-titration with *N* H_2SO_4. The product readily crystallised from the neutralised solution and after recrystallisation from water formed pale yellow needles similar to those of the trimethyl derivative and melting at almost the same temperature, 203°. It contained only two methoxyl groups, gave a reddish brown colour reaction with $FeCl_3$ and a M.P. depression of 25° when mixed with the trimethyl derivative. (Found: C, 52·94; H, 4·61 %. $C_{10}H_{10}O_6$ requires: C, 53·08; H, 4·46 %.) The dimethylpuberulic acid thus prepared is not identical with that described by Birkinshaw and Raistrick [1932].

A hypothetical substituted benzenemonocarboxylic acid with a tautomeric aldehyde group could explain these observations. However it was not possible to detect an aldehyde group. Attempts to isolate and characterise a Schiff's base, hydrazone, phenylhydrazone, *p*-nitrophenylhydrazone or semicarbazone were unsuccessful. The Angeli-Rimini test for aldehyde groups (reaction with benzenesulph-hydroxamic acid, followed by $FeCl_3$) was applied to puberulic acid and its derivatives and yielded sometimes positive, sometimes negative results. Since, however, puberulic acid and its derivatives can give with $FeCl_3$ alone the deep coloration, which is characteristic of aldehyde groups in the Angeli-Rimini test, the results are not conclusive.

We next tried to demonstrate the enolic nature of puberulic acid, by applying Windaus's method of detecting a tautomeric aldehyde group. This method, first

applied by Windaus [1924] to colchicine, consists of oxidising the entire tautomeric aldehyde group with iodine and potassium hydroxide.



With puberulic acid, however, the results were unsatisfactory, although, according to Windaus [1923], the method is generally applicable to *o*- and *p*-hydroxyaldehydes. Similar negative results were given by Dakin's [1909] oxidation with alkaline H_2O_2.

The stability of puberulic acid towards neutral perhydrol was very striking. When, after heating, oxidation finally took place, a complete breakdown to CO_2 was the only detectable result. During the oxidation with chromic acid, complications arose from the formation of rather stable complex salts.

The unsaturated character of puberulic acid is shown by a distinctly positive Baeyer's test with permanganate solution as well as by a yellow coloration with tetranitromethane [Werner, 1909], and on the addition of bromine in alcoholic solution. The typical $FeCl_3$ reaction of puberulic acid disappears after bromination.

Catalytic hydrogenation of diacetylpuberulic acid (in stabilised glacial acetic acid with platinum oxide, according to Adams) showed a hydrogen uptake of about $6H_2$. The isolation of the oily products of hydrogenation was difficult, largely owing to the very small amounts of starting material we possessed. Free puberulic acid seemed to be much less suitable for catalytic reduction. Gradual hydrogenation with Pd and then Pt led to an uptake of hydrogen to an indeterminate extent. More certain results were obtained from the hydrogenation of tetramethylpuberulic acid in stabilised glacial acetic acid with platinum oxide. The oily end-product gave the following analytical figures: C, 58·3, 58·44; H, 8·87, 8·67; OCH_3, 35·0, 34·92 %. $C_{12}H_{22}O_5$ requires: C, 58·55; H, 8·94; $3OCH_3$, 37·8; $4OCH_3$, 50·4 %. The figures for methoxyl deserve special attention. They represent strong evidence for a removal of an oxygen-containing group apart from a mere addition of hydrogen. The equation

$$C_{12}H_{14}O_6 + 10H \rightarrow C_{12}H_{22}O_5 + H_2O$$

must therefore include the disappearance of one methoxyl group without the loss of CH_3. The theoretical explanation is rather difficult.

The yellow acid $C_8H_4O_6$.

Attempts to transform one acid into the other have, up to now, been unsuccessful, and there is no experimental evidence for the supposition (suggested by the formula) that the yellow acid is a quinonoid body closely related to puberulic acid[1].

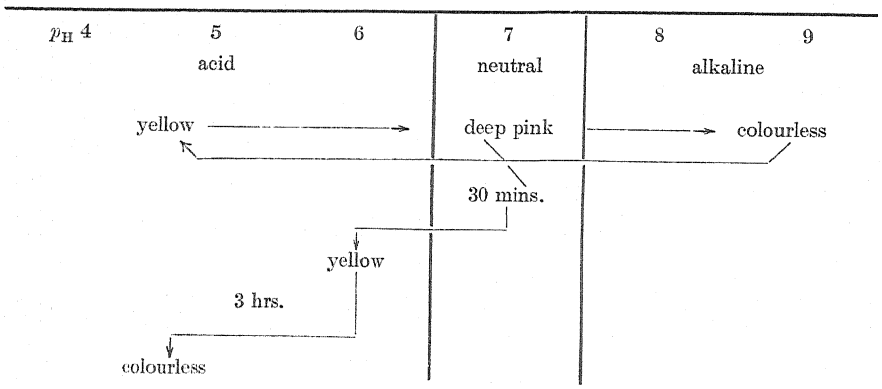
The yellow acid is not decarboxylated by the usual methods, nor can it be acetylated. Experiments for the preparation of methyl or ethyl esters yielded small amounts of crystalline products, but the analytical figures of these products did not fit with any value theoretically possible for esters of the yellow acid, although they fitted well with values for esters of puberulic acid. It thus

[1] While this note was in the press Dr H. Hoyer has obtained such evidence in my laboratory. G. B.

appeared that the yellow acid used had not been free from admixed puberulic acid. Purification of the yellow acid by recrystallisation was difficult. Only very few solvents, *e.g.* nitrobenzene or ethyl acetoacetate, were of any use.

No typical aldehyde derivatives, such as Schiff's base, phenylhydrazone or semicarbazone, could be isolated. Zinc dust distillation of the yellow product in ordinary air gave a yellow sublimate of apparently unchanged material. In an atmosphere of hydrogen however an oily substance (which later solidified) was obtained in minute yield. After purification, this product had a phenolic odour and gave a positive Baeyer's test for double bonds but no distinct $FeCl_3$ reaction; on exposure to air it became violet.

Very interesting conditions were found on neutralising the yellow acid. The yellow colour of an aqueous solution of the acid passed into a deep pink on neutralisation, and all colour disappeared in an alkaline solution. Re-acidification produced the original yellow colour but without the pink intermediate colour. The latter appeared when this re-acidified solution was again neutralised. The play of colours was reproducible several times. A neutral pink solution, on the other hand, lost all pink colour on standing for about 30 mins. and became yellow, which colour disappeared eventually after a few hours, by which time the solution had become acid. These changes are shown diagrammatically below.



Micro-titrations gave the following figures for basicity: direct titration of an aqueous solution of the acid, 1·9. Titration of a neutralised solution (calculated for a dibasic acid) which, however, reacted acid, 2·3. Back-titration of alkaline solutions, depending upon the speed of the titration itself, figures of 2·9, 3·3, 3·5.

These phenomena closely resemble those shown by pseudo-acids. If the yellow acid is quinonoid, a quinhydrone in Willstätter's sense must be considered.

Summary.

(*a*) *Puberulic acid.*

1. The method of isolation has been improved.
2. The substance is a monocarboxylic acid and can be decarboxylated.
3. Various methods have failed to reveal an aldehyde group, actual or potential.

(*b*) *Yellow acid.*

1. No aldehyde group could be demonstrated; no esters or acetyl derivatives were obtained.

2. On neutralisation the acid undergoes a curious series of colour changes, behaviour suggestive of a pseudo-acid.

We are indebted to the Moray Fund of this University as well as to Geheimrat Duisberg of Leverkusen for financial help to one of us (O. D.).

REFERENCES.

Birkinshaw and Raistrick (1932). *Biochem. J.* **26**, 441.
Dakin (1909). *Amer. Chem. J.* **42**, 477.
Damm and Schreiner (1882). *Ber. deutsch. chem. Ges.* **15**, 556.
Werner (1909). *Ber. deutsch. chem. Ges.* **42**, 4324.
Windaus (1924). *Liebig's Ann.* **439**, 59.
—— (1923). *Ber. deutsch. chem. Ges.* **56**, 846.

IV. FAT METABOLISM IN VITAMIN A DEFICIENCY: THE BLOOD-SERUM ESTERASE.

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The blood-serum of all animals investigated contains no true fat-splitting enzyme but contains enzymes capable of hydrolysing in greater or less degree the esters of the lower fatty acids, of which the mono- and tri-butyrins have been the esters chiefly used in experimental work.

Much knowledge has accumulated concerning the source, distribution and function of these esterases, but it is impossible to summarise it briefly for the conclusions reached are manifold and often contradictory. It has been suggested that they play a specific rôle in hydrolysing the lower fatty esters in the blood prior to their oxidation, but the esterases are widely distributed in all tissues, and therefore the most plausible hypothesis is that the serum esterases are derived from the blood and tissue cells, and as such have no specific function distinct from that of the tissue esterases generally. On this hypothesis variations in the serum esterase activity are an indication of variations in the esterase content of the tissues as a whole, or of the liver, which by its size and richness in esterase probably constitutes the main source of supply to the blood stream. The work of Kameo [1932], in which it was shown that X-ray irradiation of the liver caused a marked fall of the serum esterase content within 24 hours whereas direct irradiation of the blood had no effect, supports this theory. Observations on the tissue and serum esterases in the sarcoma-bearing rat [Green, 1934] also indicate a parallelism between the blood-serum esterase content and that of the liver, lung and kidney.

The esterase content of the serum can be affected by many factors. An increase has been observed after feeding large amounts of fat [Abderhalden and Rona, 1911; Greene and Summers, 1916], on a raw soy bean diet [Howarth and Chang, 1926], after starvation [Aguma, 1924] and after the injection of pilocarpine [Prewitt, 1925]. A decrease has been noted in conditions of severe intoxication with damage to cell metabolism [Fanconi, 1924; Caro, 1920], in lipaemia [Sakai, 1914; Fanconi, 1924], in starvation where Greene and Summers [1916] found a later rise above normal, and in the later stages of scurvy [Palladin and Normark, 1924]. Jobling and Bull [1912] found an increase in the serum esterase in immune haemolytic sera, whilst Bach [1922] found that it was independent of the type of food even when there was much fat in the diet.

The following work was undertaken primarily to determine the effect of vitamin A deficiency on the serum esterase content and the relation between this latter factor and the spontaneous infections arising in this deficiency disease.

Experimental.

The following method of estimating esterase activity was finally used after a trial of more complicated methods, since it proved efficient enough for the purpose in hand, which was a purely comparative one. Duplicate readings were reasonably close (± 2 %).

To 20 ml. of $M/80$ phosphate buffer of p_H 8·0 measured into a 100 ml. Erlenmeyer flask, 0·25 ml. phenolphthalein solution and 0·25 ml. of ester are added and the flask is brought to 37° by gentle heat; 0·5 ml. of blood-serum is then added and the flask incubated at 37° for 2 hours. The flask is then rapidly cooled and the contents are immediately titrated against $N/100$ NaOH until a pink tint appears matching that obtained with the blank control. The control flask contains no serum but is otherwise similar. In earlier experiments serum heated at 80° for 30 mins. to destroy the esterase was added to the control flask, but as this made an insignificant difference it was discontinued.

Continual shaking of the flask during the experimental period is recommended by most workers. Since it was observed, however, that this procedure produced a slight but definite diminution, varying with the rate of shaking, in the rate of hydrolysis, in the present work the flasks were not shaken. No antiseptic was added, as it was found that significant bacterial contamination did not occur over the experimental period of 2 hours.

The substrates used in the majority of experiments were ethyl butyrate and tributyrin. Similar results were obtained using ethyl acetate, phenyl acetate and triacetin as substrates, but as only a few determinations were made they are not reproduced.

Most of the experiments were carried out using young white rats from which blood was obtained by severing the vessels of the neck after previous stunning with a head blow. The blood was immediately centrifuged and the serum used within a maximum period of 6 hours. Blood was obtained from dogs by bleeding from an ear vein.

A standard basal diet [Green and Mellanby, 1928], deficient in vitamin A, was given to the experimental rats. The control rats received a supplement of some source of vitamin A, cod-liver oil (5 mg. daily), dried cabbage (0·5 g. daily) or carotene (0·02 mg. daily) being used. In some cases an ordinary stock diet was given but no significant differences in the serum esterase contents of the control animals were found whatever the source of vitamin A given. All rats received a supply of vitamin D in the form of "radiostol" (5 mg. daily).

In all cases the degree of hydrolysis, under the defined conditions, is expressed in terms of ml. $N/100$ NaOH required to neutralise the fatty acid liberated. The amount of esterase in normal rat-serum appears to vary within fairly wide limits. In 61 young rats, reared on complete diets, figures for ethyl-butyrase varying from 41 to 18 were obtained. The upper range was exceptional and the lower range was more often reached, but in the majority of cases values between 25 and 30 were obtained. The average figure for 30 control rats in the present series was 27·4 (Table II). Rats from the same family and from groups reared on the same diet and under similar conditions tended to give similar values. The serum content of tributyrinase appeared to be less variable; 10 control rats gave an average figure of 10·7 (Table II). It will be noted that rat-serum hydrolyses ethyl butyrate about three times as rapidly as tributyrin, and ethyl butyrate was therefore the substrate of choice where the amount of serum available made the use of only one substrate possible.

Criteria of vitamin A deficiency.

For the purpose of analysing the results it was necessary to formulate rough standards for the estimation of the degree of deficiency and infection in the vitamin A-deficient rat. The large individual variations which occur in rats on the same diet are well known; some die shortly after the decline period has set in whilst many survive for 2–3 weeks, the survival time depending to a great extent on the maximum weight reached before the decline commences. Neither the degree of xerophthalmia nor the severity of infection nor the loss in weight from the maximum reached is an absolute indication of the degree of deficiency. A sudden rapid fall of weight with an early death may be associated with only mild infection though the animal presumably died from rapid exhaustion of its vitamin A reserves, whilst a gradual fall in weight may be associated with a more severe infection which has had time to develop to the stage of more obvious lesions. In the former case the rat may be killed shortly before impending death and therefore at the last stage of deficiency, and in the latter case the rat killed at the same time may be in a definitely earlier stage of deficiency. All three criteria of deficiency have therefore been used, but it is obvious that no such clinical method of assessment can be exact. The results have however been averaged over many determinations, and the groupings should be reasonably accurate.

The criteria used for assessing the degrees of infection and deficiency are given below.

Degree of infection	Criteria.
+	Small tongue abscess and/or mild infection elsewhere (*e.g.* nasal sinuses)
+ +	Large tongue abscess or other definitely infected focus (*e.g.* lung, genito-urinary tract)
+ + +	Multiple infected foci
Degree of vitamin A deficiency	
+	Very slight xerophthalmia and/or constant weight for about 7 days or slight fall in weight for a few days
+ +	Infection (+) and/or mild xerophthalmia and/or constant weight for about 14 days or gradual fall for about 7 days
+ + +	Infection (+ +) and/or advanced xerophthalmia and/or weight falling gradually for about 14 days
+ + + +	Infection (+ + +) and/or very rapid loss in weight and/or very severe xerophthalmia (panophthalmitis)

Results.

In earlier experiments the amount of hydrolysis in normal saline, instead of buffer solution, over a period of 18 hours was determined. The results obtained with 23 deficient and 17 control rats and 7 rats on a complete but quantitatively restricted diet were proportionately similar to those obtained in the later work and are not therefore given in detail.

Typical experiment. The results of one typical experiment are given in Table I in order to show the general plan of the experiments from which the average results were derived.

Table I. *Illustrative experiment.*

Days on diet	Degree of deficiency	Degree of infection	Serum esterase: Ethyl butyrate (ml. *N*/100 NaOH)	Serum esterase: Tributyrin (ml. *N*/100 NaOH)	Weight curve
I. Basal diet alone:					
27	None	None	30·0	9·7	Rising
48	+	None	18·3	8·0	Rising very slowly
55	+	None	19·8	7·5	Rising very slowly
70	+ +	+	14·1	5·4	Plateau—20 days
59	+ +	+	9·5	4·8	Falling—10 days
59	+ + +	+ +	7·2	3·9	Falling—10 days
59	+ + + +	+ + +	2·5	2·9	Falling rapidly—6 days
II. Basal and cod-liver oil (5 mg.). Food intake restricted.					
27	–	–	30·2	10·3	Rising
48	–	–	30·8	10·1	Rising
55	–	–	22·1	8·4	Plateau—7 days
55	–	+	19·9	7·2	Falling slowly—12 days
70	–	–	24·5	8·3	Falling slowly—30 days
70	–	–	23·8	8·5	Falling rapidly—10 days
III. Basal and excess cod-liver oil (3 ml.).					
27	–	–	47·9	11·3	Falling slowly—14 days
48	–	–	36·1	10·8	Rising slowly
59	–	–	42·8	10·6	Rising slowly
70	–	–	42·9	11·2	Rising slowly
IV. Basal and excess olive oil (3 ml.) and cod-liver oil (5 mg.).					
27	–	–	31·2	.	Plateau—6 days
27	–	–	28·4	.	Slight fall—4 days
48	–	–	27·0	.	Rising slowly
70	–	–	26·8	12·6	Rising slowly
V. Basal and ethyl butyrate (0·25–1·0 ml.) and cod-liver oil (5 mg.).					
27	–	–	45·0	.	Rising
27	–	+	18·3	.	Rising
48	–	–	19·4	8·0	Falling slowly
70	–	–	26·7	10·2	Falling slowly

Rats from the same family, of the same sex and of roughly similar weight were distributed as equally as possible in the different groups. In the experiment tabulated in Table I, 5 groups of rats received the following diets:

I. The basal diet, deficient in vitamin A; intake unrestricted.

II. The basal diet and cod-liver oil (5 mg. daily). Average amount of diet per rat restricted to average amount per rat consumed by rats of group I.

III. The basal diet and a relatively large amount of cod-liver oil (3 ml. daily). Intake unrestricted.

IV. The basal diet and an amount of olive oil equivalent to the cod-liver oil given to group III (3 ml.). Cod-liver oil (5 mg. daily). Intake unrestricted.

V. The basal diet and ethyl butyrate (0·25 ml. daily for 27 days, 0·5 ml. 27–48th day and 1 ml. 48–70th day). Cod-liver oil (5 mg. daily). Intake unrestricted.

The results of several experiments planned on similar lines have been averaged and the results in this one experiment are not therefore discussed further. They serve to indicate the type of individual variation in the serum esterase content met with in all experiments.

Average results. The results obtained in 47 rats at various stages of vitamin A deficiency, in 30 which had received a complete diet and in 20 which had re-

ceived a complete diet but restricted in amount to that consumed by the rats on the deficient diet, are summarised in Table II.

Table II. *Serum esterase content of rat-serum at progressive stages of vitamin A deficiency. Average results.*

		Substrate			
Degree of deficiency	No. of rats	Ethyl butyrate (ml. *N*/100 NaOH)	No. of rats	Tributyrin (ml. *N*/100 NaOH)	Diet
0	30	27·4	10	10·7	Complete. No restriction of intake
0	20	25·0	7	8·4	Complete. Intake restricted
+	10	20·4	5	7·5	Basal
+ +	10	11·6	5	4·3	Basal
+ + +	20	7·3	6	4·1	Basal
+ + + +	7	2·5	3	2·9	Basal

It will be seen that there was a progressive fall in the serum esterase content of the rat on a diet deficient in vitamin A, which commenced at the time when the rat ceased to grow and reached a very low figure near the time of spontaneous death. The rats in the last stage of deficiency (+ + + +) were almost moribund, and, as it was considered probable that animals on the verge of death from any cause would give similar figures, only a few determinations were made. Excluding this group, there was a fall in the serum esterase content from 27·4 to 7·3 when the substrate was ethyl butyrate and from 10·7 to 4·1 with tributyrin. When loss of weight was produced by a quantitative restriction of a complete diet the average serum esterase content was only slightly below the normal. In this group individual results varied more widely from the normal than is usual. In some instances very low values were obtained but in such cases evidence of infection was always found, and these values were rejected in averaging the results.

Evidence was obtained that the serum esterase content returns to normal if the vitamin A-deficient rat is given vitamin A. Small doses of cod-liver oil were given to 6 rats at the 2nd (+ +) or 3rd (+ + +) stages of deficiency, and they were killed individually at intervals of one week. Except in one case the serum esterase content showed a progressive rise, the rat killed at 6 weeks giving a value within the normal range. The exception gave a low figure (10·5) at the 5th week and was found still to have infected foci.

Vitamin A deficiency in dogs.

In a much smaller series of dogs[1] it was found that vitamin A deficiency affected the serum esterase content in a similar manner to that observed in rats.

Table III. *Serum esterase content in vitamin A-deficient dogs. Average figures.*

		Substrate	
Diet	No. of dogs	Ethyl butyrate (ml. *N*/100 NaOH)	Tributyrin (ml. *N*/100 NaOH)
Basal and vitamin A	8	6·5	23·5
Basal alone	7	3·5	10·7

[1] Prof. E. Mellanby kindly allowed me to obtain blood samples from his dogs.

As only a relatively few animals were investigated no attempt has been made to classify the degree of deficiency. When blood samples were taken all the deficient dogs showed nervous symptoms such as incoordination of gait; some had conjunctivitis but all were fairly active. The results are summarised in Table III and it will be noted that in the dog the tributyrinase content of the serum is much greater than that of ethyl butyrase. Vitamin A deficiency has obviously produced a marked fall in the serum esterase content.

Effect of vitamin B deficiency.

Though loss of weight alone did not appear to affect the serum esterase it was interesting to determine whether another vitamin deficiency disease would bring about similar changes to that observed in vitamin A deficiency. To this end rats were given a diet deficient in the vitamin B complex; a diet which consisted of the basal vitamin A-deficient diet was used from which the chief sources of the B vitamins (marmite and lemon juice) were omitted. Each rat was given cod-liver oil (10 mg. daily) as a source of vitamin A. On this diet growth ceased very rapidly, and decline in weight set in within a few days in rats weighing 70–100 g. The average serum esterase contents of two groups of rats in which loss of weight had occurred as a result of feeding this diet are given in Table IV.

Table IV. *Serum esterase content of vitamin B-deficient rats. Average results.*

Diet	No. of rats	Substrate: Ethyl butyrate (ml. *N*/100 NaOH)	Substrate: Tributyrin (ml. *N*/100 NaOH)	Weight curve
Basal + vitamin B	7	32·6	11·3	Rising
Basal alone	12	27·8	11·1	Falling—4–10 days
Basal + vitamin B	6	24·5	—	Rising
Basal alone	7	21·1	—	Falling—10–21 days

The basal diet was the vitamin B-deficient diet described in text. Vitamin B was given in the form of dried brewer's yeast (1·0 g. daily).

Under these conditions there was only a slight fall in the serum esterase content though the loss of weight was roughly similar to that observed in vitamin A-deficient rats at the 2nd (+ +) and 3rd (+ + +) stages of deficiency at which there was a decided fall in the serum esterase content.

Effect of infection.

In view of the frequency of infection in vitamin A deficiency as compared with other deficiency diseases the question arose as to how far the effects described could be due to infection itself apart from the fundamental metabolic disorder. The serum esterase content was therefore determined in a number of rats suffering from spontaneous infections or in others in which infection had been induced by inoculation with material derived from infected lesions in vitamin A-deficient rats. All these rats received ample vitamin A. The type of spontaneous infection usually observed was an acute enteritis which may occur in epidemic form in the rat, though their diet is complete. The results have been classified together, as the type or source of the infection did not appear to affect the response in the serum esterase content. It will be observed from the summarised results given in Table V that infection itself produces a distinct

fall in the serum esterase content, though the fall is not so pronounced as in the later stages of vitamin A deficiency.

Table V. *Effect of infection on serum esterase in rats on a complete diet (ethyl butyrate as substrate).*

Degree of infection	No. of rats	Serum esterase (ml. N/100 NaOH)
0	8	26·2
+ +	8	15·0
+ + +	5	9·9

It is obvious therefore that infection is an important factor in producing the serum changes observed in vitamin A deficiency. An analysis of the lesions found in 47 rats at various stages of vitamin A deficiency (see Table II) showed, however, that 13 of these showed no macroscopic signs of infection, and yet the average figure for the serum esterase content was 13·9. It is possible that infection sufficient to affect the serum esterase level is present before definite localisation of infection occurs, but experience with experimental infections does not suggest that a mild infection would produce the degree of change actually observed. Moreover in some rats in which rapid loss of weight had occurred and in which only slight or no signs of infection were present, a fall in the serum esterase content to very low levels was observed. It is therefore probable that the metabolic disturbance associated with vitamin A deficiency is responsible for the serum change, and that infection plays a secondary part in a greater or less degree.

Effect of excess vitamin A.

Since a deficiency of vitamin A produced a diminution of the serum esterase activity it was an obvious step to determine the effect of feeding relatively large amounts of vitamin A. Rats were therefore given the basal diet and amounts of cod-liver oil of 2 or 3 ml. daily. Control rats were given similar amounts of olive oil and a third group was given ethyl butyrate in an amount of 0·25 ml. gradually increased to 1 ml. daily. The results are summarised in Table VI.

Table VI. *The effect of supplementing a complete diet with relatively large amounts of cod-liver oil, olive oil or ethyl butyrate on the esterase content of rat-serum. Average results.*

		Substrate		
Supplement to complete diet	No. of rats	Ethyl butyrate (ml. N/100 NaOH)	No. of rats	Tributyrin (ml. N/100 NaOH)
None	10	27·6	5	8·5
Cod-liver oil (2–3 ml. daily)	8	46·1	3	11·0
Olive oil (3 ml. daily)	6	28·3	—	—
Ethyl butyrate (0·25–1·0 ml. daily)	6	27·4	3	9·1

Complete diet: basal vitamin A-deficient diet + cod-liver oil (5 mg. daily).

In all of 10 rats given the large amount of cod-liver oil the capacity of the serum to hydrolyse ethyl butyrate has significantly increased. One such experiment is given in more detail in Table I and here it will be observed that there is little or no associated rise in the serum content of tributyrinase. The exact stage at which the increase occurs has not been determined; it was evidently at almost its maximum extent at the 27th day of feeding and even at the 125th day no significant change from this level was observed. The highest figure

obtained under these feeding conditions was 59·6 and the lowest 36·1; the majority gave figures between 40 and 50. No rat in the olive oil group showed this effect; the serum esterase content remained at the normal level. Only one of six rats in the ethyl butyrate group showed a rise to above normal and experiments now in progress show a tendency for a fall to occur under these conditions rather than a rise.

The rise in the serum esterase content was also observed in a few adult rats which had received excessive amounts of cod-liver oil (progressively increased up to 50 % of the diet), but where a chronic lung infection had supervened there was actually a definite fall to below normal.

No significant alteration in the serum esterase content was observed in 3 rats which received excessive doses of irradiated ergosterol (10,000 units daily) as a supplement to a normal diet for 3–5 weeks.

The rise in the serum esterase content due to feeding relatively large doses of cod-liver oil was obviously not due to an increased fat intake since a similar dose of olive oil had no effect. It is not however certain as yet whether the effect is due to excessive amounts of vitamin A. Work is in progress to determine the effects of feeding very potent vitamin A concentrates and large doses of carotene as a possible method of deciding this point.

The effect of adding vitamin A to serum *in vitro* has been determined, though a positive effect was not to be expected. Vitamin A and carotene emulsions, stable over the experimental period, were formed by adding one drop of an alcoholic solution of a very potent vitamin A concentrate or of a saturated alcoholic solution of carotene to the buffer mixture. A similar amount of alcohol was added to the control flasks. In three determinations using both normal serum and serum from vitamin A-deficient animals no increase but a slight decrease was observed in the degree of hydrolysis of ethyl butyrate. Carotene was without effect in a similar number of experiments.

Discussion.

It has been shown that vitamin A deficiency brings about a pronounced fall in the esterase content of the blood-serum. It is obvious from previous work on the subject and from my own findings that such a fall is not a change peculiar to vitamin A deficiency; amongst other causes infection and, in the rat, inoculation with sarcoma tissue [Green, 1934], have been observed. There is some reason therefore for considering it a secondary change and one unlikely to throw direct light on the physiology of vitamin A. The fact however that a similar change does not occur, except in a very much less degree, in other conditions producing loss of weight (underfeeding, vitamin B deficiency), suggests that the change may have some significance in the pathological processes due to vitamin A deficiency. Infection probably plays a part as it certainly produces a fall in the serum esterase content both in animals and in man [Green, 1934], but it has already been indicated that this is not the primary cause. Several workers have attempted to identify serum esterase with serum complement. There appears to be a parallelism between the contents of these two factors in the serum of different animals, according to Jobling and Bull [1913] but Gordon and Wormall [1929] have shown that whilst the complement and esterase contents of the serum tend to run parallel there are occasional discrepancies, and they were able to show that there was a significant amount of esterase in serum which had been heated to the extent necessary to destroy all the complement. In view however of the quantitative association of these two factors it is

interesting to compare the present results with those obtained by Osborne [1931] on the serum complement of vitamin A-deficient rats. He found a tendency towards a lowered serum complement content in vitamin A deficiency in the rat, though it is not stated at what stage of the deficiency the observations were made. It is tentatively suggested that this fall may be responsible for the lowered resistance of the vitamin A-deficient rat to infection. If there is a quantitative relationship between the serum esterase and complement it seems possible that the decrease in complement may be the result rather than the cause of infection, for the present findings show that inoculated infection produces a very definite reduction in the serum esterase content.

Future work may show that a fall in the serum esterase content occurs in many conditions in which there is a profound metabolic disturbance in the body cells. Until we know more in this connection and in regard to the function of the esterases in general, and the serum esterase in particular, it may be impossible to assess the real significance of the present findings.

Summary.

1. Vitamin A deficiency in the rat produces a large and progressive decrease in the esterase content of the blood-serum.

2. The infected rat on a complete diet also shows a considerable fall in the serum esterase content, but in rats which have lost weight on account of underfeeding or deficiency in the vitamin B complex, there is, on the average, only a very slight fall.

3. Relatively large amounts of cod-liver oil in the diet produce a rise in the serum esterase content well above normal.

4. The relation of these findings to infection in vitamin A deficiency is discussed.

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REFERENCES.

Abderhalden and Rona (1911). *Z. physiol. Chem.* **75**, 30.
Aguma (1924). *J. Biochem.* **4**, 239.
Bach (1922). *Z. klin. Med.* **95**, 103.
Caro (1920). *Z. klin. Med.* **89**, 49.
Fanconi (1924). *Ferm. Forsch.* **7**, 307.
Gordon and Wormall (1929). *Biochem. J.* **23**, 730.
Green (1934). *Brit. J. Exp. Path.* (in press).
—— and Mellanby (1928). *Brit. Med. J.* ii, 691.
Greene and Summers (1916). *Amer. J. Physiol.* **40**, 146.
Howarth and Chang (1926). *Amer. J. Physiol.* **78**, 224.
Jobling and Bull (1912). *J. Exp. Med.* **16**, 483.
—— —— (1913). *J. Exp. Med.* **17**, 61.
Kameo (1932). *J. Biochem.* **15**, 229.
Osborne (1931). *Biochem. J.* **25**, 2137.
Palladin and Normark (1924). *Biochem. Z.* **152**, 420.
Prewitt (1925). *Amer. J. Physiol.* **73**, 1.
Sakai (1914). *Biochem. Z.* **62**, 387.

V. FAT METABOLISM IN VITAMIN A DEFICIENCY: THE UTILISATION OF FAT AND THE DESATURATION OF FAT IN THE LIVER.

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THE function of vitamin A in metabolism still remains obscure. Its close natural association with the fats has led some workers to search for a possible relation to fat metabolism, but so far little of significance has been found. The profound loss of body fat in the rat that has died from vitamin A deficiency is obvious on casual examination, and it has been demonstrated by quantitative methods [Amada, 1923; Rost, 1926]. An increase in the blood-fat during vitamin A deficiency was observed by Amada [1923] and Collazo and Bosch [1923]. This loss of body fat is not superficially different however from that seen in other deficiency syndromes or in other conditions producing rapid loss of weight, such as infection and starvation. Unless the rate at which the fat disappears differs essentially from that observed in animals losing a similar amount of weight from other causes it is unlikely to be of particular significance. A more detailed survey of this loss of body fat was made by Javillier *et al.* [1929], who found in the vitamin A-deficient animal a decrease in the lipoids of the skin, liver and muscle and an increase in those of the brain and bone, whilst the spleen, lung, kidney and heart were normal in this respect. There was a decrease in the cholesterol content of all organs except the skin. They produced no evidence that these changes were specific to vitamin A deficiency.

The possibility that if vitamin A were intimately concerned with fat metabolism a large amount of fat in the diet might accelerate the onset of deficiency symptoms was explored by Drummond [1919], who found that in adult rats the addition of fat to the diet had no effect of this kind. It was shown that the adult rat on a vitamin A-deficient diet could absorb large quantities of fatty acids and effect their synthesis into fats over a period of 3 months. In view however of the now well-known fact that the adult rat may possess sufficient reserves of vitamin A in its liver to maintain it for a year or more, these early experiments cannot be considered conclusive.

The present work was designed as a preliminary attempt to investigate fat metabolism in vitamin A deficiency. Three lines of enquiry were pursued. (1) Would a large increase in the fat content of the diet produce a more rapid utilisation of the vitamin A reserves of the body? (2) Does the loss of body fat in the vitamin A-deficient animal differ quantitatively from that due to loss of weight produced by a quantitative restriction of a complete diet? (3) Is the process of fatty acid desaturation in the liver, as indicated by its content of unsaturated fatty acids, of a different order from that observed in loss of body fat due to under-feeding or infection?

The experimental results obtained in investigations covering all these points are now considered.

Experimental.

Young albino rats, raised in this laboratory, were used, and for comparative groups rats of roughly similar weight and the same sex were included in equal numbers.

Two diets were used, one a standard basal vitamin A-deficient diet [Green and Mellanby, 1928], used in this laboratory for some years, which contains roughly 8 % of fat, the other a high fat diet, containing roughly 70 % of fat, with the following composition:

Vegetable margarine	59·0 %
Olive oil	11·0
Caseinogen	18·5
Marmite	7·5
Salt mixture	4·0

The margarine was a high-class brand, consisting largely of a semi-hydrogenated vegetable fat with a small percentage of liquid vegetable oil. It was irradiated in a thin melted layer from a mercury vapour lamp at 8″ distance for 12 hours with the idea of destroying any possible trace of vitamin A. The unsaponifiable fraction was negative to the $SbCl_3$ test for vitamin A.

The olive oil was heated in a current of air at 120° for 6 hours.

The caseinogen was heated in air at 120° for 36 hours.

The McCollum salt mixture (185) was used.

In order to avoid a possible partial deficiency of vitamin B all the rats on the high fat diet received an additional supply in the form of dried brewer's yeast (0·5 g. daily). All rats used in this work received a supply of vitamin D in the form of "radiostol", 3 drops weekly[1].

The crude fatty acid content of the body was determined, after removal of the liver and alimentary tract, by saponification with alcoholic potash, conversion of the soaps into free fatty acid by treatment with sulphuric acid and extraction with light petroleum. The petroleum solution was evaporated to dryness and the fatty acids weighed.

The iodine value of the crude fatty acids of the liver was determined by the method of Wijs following in detail the procedure described by Leathes [1913].

Survival time on the high fat diet.

Rats given the high fat diet ran the typical course of animals deficient in vitamin A, developed xerophthalmia and at autopsy showed the typical infected lesions. The addition of vitamin A to this diet in the form of cod-liver oil (10 mg. daily) or dried cabbage (0·5 g. daily) allowed of normal growth and development in a control group of rats. A group of 15 young rats was given the high fat diet and a control group of 15 was given the standard basal diet. Rats dying before the onset of deficiency symptoms were discarded in analysing the results which are summarised in Table I.

Table I. *Effect of a high fat diet, deficient in vitamin A, on the growth and survival time of the rat.*

Diet	Fat content of diet %	No. of rats in group	Average survival time days	Average maximum increase in wt. g.
Basal	8	12	60	69
High fat	70	11	72	33

[1] It was presumed that after prolonged irradiation little vitamin D would remain in the margarine of the high fat diet.

The growth rate of the rats on the high fat diet was roughly only half that of the control group. Since it is possible that a slower rate of growth resulted in a lessened demand for vitamin A, it becomes impossible to draw any absolute conclusion from the experiment. The high fat group survived for a slightly longer period, on the average, than the control group, which may quite possibly have been due to the slower rate of growth or less possibly to the fact that the high fat diet, though obviously very deficient in vitamin A, contained slightly more than the control basal diet. The experiment on these lines was not however continued further, for the preliminary result appeared to show quite clearly that an excessive amount of fat in the diet had not made excessive demands on the vitamin A reserves such as might be expected if vitamin A were directly related to the metabolism of the fatty acids. If such were not the case the survival time of the high fat group would presumably have been much less than that of the basal group, whereas it was slightly longer.

Loss of body fat in vitamin A deficiency.

Three groups of young rats were given the basal, low fat diet. One group (Table II (1)) received vitamin A in the form of cod-liver oil (10 mg. daily) and the amount of diet eaten was not limited; a second group (2) received a similar diet, but the amount ingested was restricted to the average amount consumed by a third group ((4), (5) and (6)) which received no vitamin A. Rats receiving vitamin A were killed at times corresponding with the spontaneous deaths of rats receiving the basal diet alone. A number of rats ((4) and (5)) in the third group (not receiving vitamin A) were killed during the earlier stages of deficiency symptoms. The remainder of this group were continued on the diet until death occurred. Two other groups of rats ((3) and (7)) were given the high fat diet, with or without the addition of vitamin A. The rats in the group receiving vitamin A were killed at times corresponding with the spontaneous deaths of the vitamin A-deficient group.

Estimations of the total body fat were made in all cases. The results are summarised in Table II.

Table II. *The effect of vitamin A deficiency compared with that of restriction of total food intake on the fat content of the rat.*

Diet	No. of rats in group	Average wt. at death g.	Weight curve	Total body fat %	Remarks
+ vitamin A.					
(1) Basal	9	108	Rising	10·8	Killed when rats of group (6) died
(2) Basal	9	68	Falling rapidly	3·5	Food intake restricted to that of group (6). Killed when rats of group (6) died
(3) High fat	6	125	Rising	17·2	Killed when rats of group (7) died
No vitamin A.					
(4) Basal	8	84	Level	9·0	Killed at early stage of vitamin A deficiency
(5) Basal	5	79	Falling slowly	7·0	Killed at later stage of deficiency—7 days later (on average) than group (4)
(6) Basal	9	64	Falling rapidly	2·6	Spontaneous death. Terminal stage of deficiency
(7) High fat	6	54	Falling rapidly	2·8	Spontaneous death. Terminal stage of deficiency

It will be noted that in the early stage of vitamin A deficiency (Table II (4)) there was a slight fall in the amount of body fat, and that this continued to fall gradually with the gradual loss in weight (5). With the rapid loss of weight which closely preceded death the loss of body fat was very rapid. It is to be noted however that this loss was not significantly greater than where the loss of body fat was due to a simple restriction in the amount of food eaten (*cf.* (2) and (6)).

As would be expected the rats receiving the high fat diet had more body fat than those of the group receiving the standard basal diet when, in each case, vitamin A was given (*cf.* (1) and (3)), but it is interesting to note that in the absence of vitamin A the total body fat reaches a similar low level in each group at the time of death (*cf.* (6) and (7)). There is not the slightest indication here of a failure of the animal, deficient in vitamin A, to utilise its available fat reserves to the fullest extent. Loss of weight whether produced by underfeeding or by vitamin A deficiency has resulted in a mobilisation and presumably the oxidation of the greater part of the fat originally present in the body before the decline in weight set in.

The iodine value of the liver fats in vitamin A deficiency.

It seemed desirable to find whether the first stage of fat metabolism, that of desaturation in the liver, differed in the course of vitamin A deficiency from that observed in the underfed animal, where the iodine value of the liver fat rises as the amount of fat in the liver falls. To this end groups of rats similar to those of the preceding section were used with an additional group, receiving a complete diet, in which the amount of liver fat was reduced by artificial infection with a pathogenic strain of haemolytic streptococci or pathogenic organisms of

Table III. *The effect of vitamin A deficiency, underfeeding and artificial infection on the iodine value of the liver fatty acids.*

Diet	Degree and type of infection	No. of rats in group	Liver (average values) Weight g.	Fat content %	Iodine value of fat	Remarks
+vitamin A.						
(1) Basal	None	17	6·3	4·1	131	Killed when rats of group (7) died
(2) Basal	None	8	3·8	2·7	161	Food intake restricted to that of group (7). Killed when rats of group (7) died
(3) Basal	Severe. Inoculated	11	4·0	2·7	169	Spontaneous death
(4) High fat	None	6	6·4	7·7	130	Killed when rats of group (8) died
No vitamin A.						
(5) Basal	None macroscopically	22	4·3	4·4	124	Killed at early stage of deficiency. Weight stationary
(6) Basal	Mild. Autogenous	10	3·0	3·9	127	Spontaneous death but only moderate loss of weight
(7) Basal	Severe. Autogenous	16	2·6	2·4	160	Spontaneous death. Rapid loss of weight
(8) High fat	Severe. Autogenous	6	2·2	3·0	169	Spontaneous death. Rapid loss of weight

the colon group. The iodine value of the liver fat was estimated in each rat. The results are summarised in Table III.

It will be noted (Table III (5)) that in the very early stages of vitamin A deficiency there was no decrease in the percentage amount of liver fat; in fact it was slightly higher than that of the control group; the iodine value of the liver fat was, as would be expected, somewhat lower than that of the control group (*cf.* (1) and (5)). Group (6) consists of rats which died as a result of vitamin A deficiency, but death was preceded by only a moderate loss in weight, and the amount of liver fat was only slightly below the normal level. This group was particularly interesting, for the iodine value of the liver fat was practically the same as that of the controls (*cf.* (1) and (6)). On the other hand, when the amount of liver fat was much reduced as a result of vitamin A deficiency (group (7)), restriction of food intake (group (2)) or infection (group (3)), the iodine value of the fat rose to roughly the same level in each case.

Group (4), which received the high fat diet and vitamin A, showed a much higher liver fat content than group (1), which received the standard basal diet and vitamin A. It is particularly to be noted that group (8), given the high fat diet without vitamin A, showed a decrease in the amount of liver fat to a level not much above that of group (7), though the amount of fat in the liver before the stage of vitamin A deficiency was reached was, in the two groups, very different.

It is clear from these results that the iodine value of the liver fat has risen as the amount of fat in the liver has fallen, a fact which Hartley and Mavrogordato [1908] first observed, and which was confirmed in human livers by Imrie [1914]. The later stages of vitamin A deficiency, underfeeding and severe infection have all produced a diminution in the amount of fat in the liver, which in effect involves the saturated more than the unsaturated fatty acids and thus results in a rise in the iodine value of the residual fatty acids. The effect of vitamin A deficiency, was, in this connection, no different from that when a diminution in the amount of liver fat was produced by other means. It would seem that the liver, devoid of vitamin A, is able to desaturate the fats presented to it, or otherwise it would be expected that fatty acids of a lower iodine value would be found in it than is the case where a loss of fat occurs in the liver containing vitamin A. Removal of fat appears to proceed quite efficiently even when the diet is very rich in fat, a fact which strongly suggests that vitamin A is not concerned with this process.

Discussion.

Though these findings throw no new light on the physiology of vitamin A, they assist, in conjunction with the findings of other workers, in excluding some of the more obvious features of fat metabolism from a probable association with vitamin A. Large quantities of fat can be absorbed in the absence of vitamin A from the diet, and mobilisation, desaturation in the liver and oxidation apparently proceed normally when the vitamin A reserves of the animal are exhausted.

If it is conjectured that vitamin A is concerned with some aspect of fat metabolism it is probably not with that of the simple fatty acids but of the more complex lipins. Monaghan and Schmitt [1932] have suggested that vitamin A is concerned with phospholipin metabolism on the grounds of experiments *in vitro* in which it was shown that both the unsaponifiable fraction of cod-liver oil and carotene inhibit the oxidation of linoleic acid. Linoleic and other more highly unsaturated fatty acids are found chiefly in the phospholipins of the body, and the preliminary results of these workers suggest a large diminution in the phospholipin content of rats deficient in vitamin A. There is however no

indication in the present results (see Table III) that, as regards the liver, there is any significant difference in the content of unsaturated fatty acids between the vitamin A-deficient rat and the rat in which loss of fat has occurred from other causes in the presence of vitamin A.

There is another discovery which originally suggested a possible connection between vitamin A and lipin metabolism. Mellanby [1926] found that in the vitamin A-deficient dog there was a widespread degeneration of nerve fibres which was readily shown by staining with agents fixed by free fat. He has suggested that this breakdown of the medullary sheath may be the primary lesion of vitamin A deficiency, the keratinisation of epithelium and infection being a secondary trophic change. The intimate structural relationship of the lipins to the nerve myelin sheath suggested to me that some alteration in their metabolism might be the factor leading to degeneration. It was later shown by Mellanby [1933], however, that this degeneration only affected the afferent nerves, whilst the efferent nerves were left relatively intact, and it does not appear probable that if the lesion were due to a chemical change in the medullary sheath it would affect one physiological type of nerve only. It is perhaps not without significance however that the presence of carotene in the nerve fibre has been demonstrated [Monaghan and Schmitt, 1930–31]. These suggestions are obviously speculative, but they indicate the possibility that though vitamin A is probably not related to any of the simpler manifestations, it may yet prove to be related to some more obscure process of fat metabolism.

Summary.

1. No evidence was obtained that a diet very rich in fat accelerates the utilisation of vitamin A in the body.

2. The diminution in the total crude fat of the whole body is not significantly different in the vitamin A-deficient rat from that in the rat on a complete diet whose food intake is restricted to that of the deficient animal.

3. A rise in the iodine value of the fatty acids of the liver, coincident with a fall in the percentage amount of fat in the liver, occurs in the end stages of vitamin A deficiency. The rise is of a similar order to that observed where a similar loss of liver fat has been produced by a restriction of food intake or by infection.

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References.

Amada (1923). *Biochem. Z.* **142**, 44.
Collazo and Bosch (1923). *Biochem. Z.* **141**, 370.
Drummond (1919). *Biochem. J.* **13**, 95.
Green and Mellanby (1928). *Brit. Med. J.* ii, 691.
Hartley and Mavrogordato (1908). *J. Path. Bact.* **12**, 371.
Imrie (1914). *J. Path. Bact.* **19**, 245.
Javillier, Rousseau and Emerique (1929). *Compt. Rend. Acad. Sci.* **188**, 580.
Leathes (1913). The fats. (Longmans, Green and Co.)
Mellanby (1926). *J. Physiol.* **24**; *Proc.* lxi.
—— (1933). *Edinburgh Med. J.* **40**, 197.
Monaghan and Schmitt (1930–31). *Proc. Soc. Exp. Biol. Med.* **28**, 705.
—— —— (1932). *J. Biol. Chem.* **96**, 387.
Rost (1926). *Brun's Beitr. klin. Surg.* **138**, 472.

VI. RESEARCHES ON FAT METABOLISM. II.

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(1) IT was shown in the first paper of this series [Verkade *et al.*, 1933] that with two healthy subjects (V. and v. d. L.) considerable quantities of undecanedioic acid were excreted in the urine after administration of triundecylin. In the human organism the terminal methyl group of the undecoic acid, the component acid of triundecylin, is thus oxidised—and as we shall see later, very readily—to a carboxyl group. The term *ω-oxidation* was introduced by us for this type of oxidation

$$CH_3.(CH_2)_9.COOH \rightarrow HOOC.(CH_2)_9.COOH.$$

The present communication deals with the question whether the administration of chemically pure simple triglycerides of other, higher and lower, odd and even saturated fatty acids leads similarly to the excretion of the corresponding dicarboxylic acids and if so, to what extent.

Influence of simultaneous administration of carbohydrate.

(2) During our original experiments with triundecylin this formed part of a diet which was very poor in carbohydrate; the subject V., *e.g.*, then received 23 g. carbohydrate, 136 g. protein, 192 g. ordinary food fat and 100 g. triundecylin. In a continued study of the *diaciduria* (thus we term the phenomenon of excretion of dicarboxylic or dioic acids in the urine) produced by triundecylin we have discovered that addition of a liberal amount of carbohydrate to the diet leads with most people, among them also the subjects V. and v. d. L., to a pronounced rise of the dioic acid excretion. We have repeatedly taken advantage of this noteworthy and definitely important fact, which very probably points to a function of the liver in the processes here in question and which will be discussed fully in a later communication, in the investigations which will be dealt with in this paper.

Because of their general plan the simple experiments described in this paper can only serve for a preliminary orientation; we shall return to this later. For this reason also we have considered it superfluous to indicate in detail the composition of the diet administered and the distribution of it over the day for all the experiments. We have frequently regarded it as sufficient to mention only a few brief data on the point.

EXPERIMENTAL.

Tricaprylin.

(3) The tricaprylin used was prepared in the way described elsewhere [Verkade *et al.*, 1932] and refined in the way commonly employed technically. Caprylic (octoic) acid with a setting-point of 16·1°, which was isolated from coconut fat *via* the ethyl ester, served as our starting-material. The setting-point of an earlier caprylic acid specimen purified by us with the greatest

possible care, which had at that time to serve for precision measurements, lay only a little higher, namely at 16·35°; Garner and Randall [1924] give the same setting-point for a similar product. The saponification number of the fat obtained was 357·3 (calculated 357·8) and the setting-point 9·45°; we find the values 8–8·3° [Scheij, 1899], 9° [Jaeger, 1917] and 8·3° [Loskit, 1928] for the melting-point of tricaprylin in the literature.

A diet which contained 100 g. tricaprylin as well as amongst other things a liberal amount of carbohydrate, was administered to the healthy subject V. The fat was taken mixed with coffee in portions of 20 g. at practically equal intervals in the course of 9 hours; the carbohydrate was also distributed over this period in several portions. The urine of the subject was carefully collected from the time of partaking of the first portion of fat and indeed up to and including that passed immediately after rising on the next day; on the basis of researches to be published later we knew that collection of the urine for a longer time was unnecessary.

The collected urine was made strongly alkaline with KOH and then evaporated as far as possible, and the residue was heated for a considerable time on the water-bath. Hydrolysis of the hippuric acid present takes place as a result. The removal of this acid, which is relatively little soluble in cold water, facilitates the isolation in a pure state of the dicarboxylic acid sought, especially if—as, for example, in the isolation of sebacic acid discussed on p. 34—extraction with benzene is not employed; hippuric acid is only very slightly soluble in boiling benzene. It is almost certain that compounds of dicarboxylic acids with glycine, glucuronic acid *etc.* if present would also be hydrolysed. The residue after evaporation was taken up in water, acidified with hydrochloric acid and continuously extracted with ether for a considerable time. The ethereal extract was filtered and the residue obtained after distilling off the ether was dried *in vacuo*. It was subsequently extracted several times with 50 ml. of boiling benzene. The mass of crystals gradually separating from the combined cold benzene solutions contains practically all the suberic acid present; this acid is indeed practically insoluble in cold benzene [Verkade and Coops, 1930, 2]. The crystals were filtered off and taken up in hot water. The hot aqueous solution was treated with a small amount of adsorption charcoal and then filtered through a small glass filter. From this solution evaporated to about 15 ml. 0·18 g. of practically pure suberic acid (octanedioic acid) separated out on standing in the ice-chest. The M.P. of this product was 139–141°; the mixed M.P. with a specimen of suberic acid prepared in the usual way from castor oil [Verkade, 1927] and melting at 140–141° was 139–141°.

0·1009 g. required 11·79 ml. 0·0974 *N* NaOH; equiv. wt. 87·9; calc. 87·06.

Tricaprylin was the last triglyceride we examined. As appears from the table on p. 37 we had good reason to expect that only very small quantities of suberic acid would be excreted after administration of this substance [*cf.* Verkade and van der Lee, 1933]. We therefore gave the subject v. d. L.—the latter generally shows the greatest tendency to diaciduria of all subjects who have been at our disposal—100 g. of tricaprylin in a single portion together with 160 g. of bread, 25 g. of sugar and 10 g. of butter shortly after rising. Our intention in doing this was naturally to flood the body with resorbed caprylic acid—we shall not consider the question of the form in which this circulates in the blood—and thus to provoke accumulation of suberic acid in the blood and excretion thereof in the urine. The experiment with the subject V. described above has taught us later that this forced administration of tricaprylin was unnecessary. Diarrhoea occurred in the evening as was indeed not surprising;

investigation of the faeces showed, however, that by far the greater part of the fat administered had been resorbed. The urine was again collected up to the next morning while the subject was still fasting.

In this experiment 0·12 g. of practically white suberic acid, M.P. 139–140°, was isolated from the urine; the mixed M.P. was also 139–140°.

(Found: equiv. wt. 86·8, C 55·4, 55·5; H 7·7, 7·7%: calc. for $C_8H_{14}O_4$: equiv. wt. 87·06; C 55·14; H 8·11 %.)

Trinonylin.

(4) The preparation and purification of the trinonylin employed has already been described elsewhere [Verkade *et al.*, 1932]. The setting-point of this fat was 8·7°, the saponification number 328·7 (calc. 328·5).

To the subject v. d. L. a diet was administered which contained besides 100 g. of trinonylin a liberal amount of carbohydrate in the form of bread and sugar. The fat was taken as such in portions of 20 g. at nearly equal intervals in the course of 10½ hours; the carbohydrate was also taken in portions a short time before the fat. The urine was again collected up to the following morning.

The ethereal extract of the urine obtained in the way described on p. 32 and dried *in vacuo* was extracted several times with 50 ml. of boiling benzene. The odd acids of the oxalic acid series, with the exception of the two lowest members, are readily soluble in boiling benzene and are thus rapidly extracted in this way even when present in somewhat larger quantities; on the other hand they are very sparingly soluble in cold benzene; azelaic acid, *e.g.* only to the extent of 9 mg. in 100 g. of benzene at 25° [Verkade and Coops, 1930, 2]. The mass of crystals separated from the combined ice-cold benzene solutions was filtered off at the pump and taken up in hot water. The hot aqueous solution was treated with some adsorption charcoal and concentrated to about 75 ml. On cooling 0·60 g. pure azelaic acid (nonanedioic acid) separated out. The M.P. of this product was 104–105°; the mixed M.P. with a specimen of azelaic acid prepared in the usual way from castor oil [Verkade, 1927] and melting at 106·5–107·5° was 105–106°. A further 0·05 g. of the same acid was obtained from the benzene and aqueous mother-liquors. Thus a total of 0·65 g. of azelaic acid could be isolated from the urine.

(0·2020 g. required 20·53 ml. 0·1041 *N* NaOH: equiv. wt. 94·5; calc. 94·06.)

A similar experiment was made with the subject V. The latter excreted 0·35 g. of azelaic acid in the urine after taking 85 g. of trinonylin.

(Found: C, 57·4, 57·6; H, 8·3, 8·4 %; calc. for $C_9H_{16}O_4$: C, 57·41; H, 8·56 %.)

Tricaprin.

(5) The tricaprin used was prepared in the manner described elsewhere and purified by recrystallisation from a mixture of alcohol and ether. Capric (decoic) acid setting at 31·35°, which was obtained from pure *n*-octylmalonic acid, served as starting material. The fat obtained set at 30·95°, thus below body-temperature, and was consequently applicable as such to the experiments in hand (see on the other hand sections (7) and (8)); the values 31·1° [Scheij, 1899; Jaeger, 1917] and 31·0° [Loskit, 1928] are given for the M.P. of tricaprin in the literature. The saponification number of the fat was 302·7 (calc. 303·4).

The experiments were here carried out with the standard technique which we apply to other work and which will be discussed rather more fully in a later paper.

Shortly after rising 25 g. of tricaprin melted up in coffee together with a liberal amount of carbohydrate were administered to the sober, healthy subject V. The urine was collected at intervals of 1–2 hours and the presence of sebacic acid was tested for in each portion by treating the urine with a concentrated mineral acid, *e.g.* phosphoric acid; even with very small concentrations of sebacic acid the latter then separates out as micro-crystals in a characteristic way. The collection of the urine was continued with the subject still fasting until two successive portions gave a negative reaction.

The ethereal extract obtained in the manner described on p. 32 was extracted with boiling water, and the filtered aqueous solution was evaporated to about 50 ml.; on cooling practically pure sebacic acid (decanedioic acid) crystallised out. This acid weighed 0·55 g. and melted at 129·5–130·5°; the mixed M.P. with a specimen of sebacic acid prepared in the usual way from castor oil and melting at 132·5–133·5° was 132–133°.

(0·1202 g. required 11·26 ml. 0·1046 *N* NaOH: equiv. wt. 102·0; calc. 101·08.)

In another similar experiment this subject excreted 0·11 g. of sebacic acid in the urine.

Two similar experiments were made with the subject v. d. L. With the latter 1·10 g. and 0·29 g. of sebacic acid were respectively excreted in the urine after partaking of 25 g. of tricaprin together with a liberal amount of carbohydrate. Below are given some analytical data for the acid isolated in the last experiment, recrystallised once more from water after treatment with some adsorption charcoal and then melting at 131·5–133°.

(Found: equiv. wt. 101·7; C, 59·6, 55·6; H, 8·8, 8·8 %: calc. for $C_{10}H_{18}O_4$: equiv. wt. 101·08; C, 59·36; H, 8·98 %.)

Triundecylin.

(6) It is here sufficient to state that, employing the above mentioned standard technique, with the administration of 25 g. of triundecylin together with a liberal amount of carbohydrate, amounts of undecanedioic acid, varying from 0·29 g. to 0·66 g. and from 0·60 g. to 1·59 g. were excreted by the subjects V. and v. d. L. respectively. These experiments will be discussed in detail in the communication in which we deal with the rôle of the carbohydrate.

Trilaurin.

(7) The trilaurin required was obtained in a yield of more than 85 % by crystallising tangkallak fat (from *Litsaea sebifera* Bl.) several times from a mixture of 2 vols. 96 % alcohol and 1 vol. ether [van Eldik Thieme, 1911]. Its saponification number was 263·0 (calc. 263·5). The M.P. of trilaurin is 46·2°.

By melting together 1 part of trilaurin with 1 part of coconut fat a mixture was obtained which was completely liquid at body-temperature. The use of such a mixture appeared to us to be necessary to obtain a good adsorption of the fat. On administering trilaurin as such, completely melted up in coffee or thin broth, it was excreted to an appreciable extent in the faeces in the form of large and small solid pieces. For two reasons we have used coconut fat for the purpose mentioned and not a fatty oil setting only at low temperatures. Firstly it is well known in the food fat industry that the addition of coconut fat is particularly suitable for lowering the M.P. of a fat; secondly coconut fat contains amongst its component fatty acids an appreciable amount (about 50 %) of lauric acid, although in the form of mixed glycerides. Against this addition of coconut fat one can *a priori* raise the objection that this fat contains as com-

ponent fatty acids various other higher and lower acids of the same series in varying though always much smaller amounts. One may ask whether administration of the fat mixture in question might lead to the excretion of a mixture of several dicarboxylic acids in the urine, so that the isolation in the pure state of dodecanedioic acid possibly present and the proof of the origin thereof as an ω-oxidation product of lauric acid might be made much more difficult. From what follows it will be seen that this objection is of a purely theoretical nature and, taking into account the quantitative composition of coconut fat [see *e.g.* Taylor and Clarke, 1927], we get an indication why this is so. Reference may be made here to the third paper of this series.

We have administered this fat mixture to various subjects, finally, since no positive results were obtained, in very large quantities. We shall only discuss two of these experiments here, one of them somewhat more fully.

225 g. of the fat mixture in question were administered in the course of 8 hours as part of a diet very poor in carbohydrate to the healthy subject B., to whom on the preceding day no food whatever had been given. We succeeded in isolating from the urine collected up to the following morning a few mg. of a substance which melted at 118–122° and the mixed M.P. of which with a specimen of dodecanedioic acid, prepared by the method of Walker and Lumsden [1901] and melting at 125–127°, was 119–124°. After recrystallising once more from boiling benzene this substance melted at 122·5–125·5°. Taking the solubility relations of this substance also into account—it was very slightly soluble in cold water and cold benzene [see Verkade and Coops, 1930, 2], more readily in cold ether and boiling water and readily in boiling benzene—the conclusion is justified that we were very probably dealing with dodecanedioic acid. The amount of material was quite inadequate for a more definite confirmation by titration or elementary micro-analysis.

In the second experiment 190 g. of the same fat mixture were administered to the same subject in the course of $8\frac{1}{4}$ hours, together with a liberal amount of carbohydrate in the form of bread and sugar. The composition of the diet and the distribution of it over the day are indicated in the following table:

Time	Bread g.	Sugar g.	Fat mixture g.
9.15	40	8	—
9.40	—	—	30
11.00	—	—	30
11.10	40	—	—
12.15	—	—	30
13.15	80	8	—
13.30	—	—	25
14.45	—	—	25
16.45	30	—	25
18.00	—	—	25
18.55	70	—	—
	260	16	190

Each portion was taken after being completely melted up in thin broth; taking the large amount of fat was greatly facilitated by adding some lemon juice and eating a few grapes. On working up the urine, again collected until the following morning, indications of the presence of only a very small amount of dodecanedioic acid were obtained.

Notwithstanding the administration of tea and of *pulvis opii*—in the second experiment, for example, the subject received 50 mg. of the latter each time at 12 hours 30, 17 hours and 23 hours—diarrhoea occurred in both experiments,

which can indeed cause no surprise. It occurred, however, only late at night and it is therefore quite certain that a very considerable part of the fat mixture administered had been adsorbed. That no appreciable excretion of dodecanedioic acid in the urine took place, in spite of the large amount of fat adsorbed in these experiments, is very striking; for example, for comparison with the result of the second experiment it should be mentioned that the subject B. excreted a large amount (namely 1·05 g. and 0·45 g. respectively) of undecanedioic acid on the administration of only 25 g. of triundecylin together with a liberal amount of carbohydrate employing the standard technique in the tests. The same remarkable difference in behaviour between trilaurin and the lower triglycerides was observed with other subjects, among them the subject V.

Since natural products (tangkallak fat and coconut fat) served as the starting materials in the experiments discussed in this section, for the sake of completeness we shall deal with the question whether these products possibly contained preformed dodecanedioic acid in some form or other so that the traces of this acid, which were present in the urine of the subject, might be derived from this. This question is really of no importance to us. Indeed in the case of a confirmatory answer the contrast between trilaurin and the lower triglycerides, to which we have just drawn attention, becomes, if possible, still more pronounced. However, a negative answer is already clearly implied in the fact that—as will be shown in later communications partly from data in the literature [see, among others, Baer and Blum, 1908; Mori, 1918; Flaschenträger, 1927], partly from our own investigations—animal and human organisms are able to burn considerable amounts of the higher normal saturated dicarboxylic acids in a day, combined with the consideration that the trilaurin obtained by repeated crystallisation of tangkallak fat from alcohol-ether and the coconut fat (both products which were repeatedly and carefully investigated) in no case contained appreciable amounts of these acids.

Tritridecylin.

(8) The preparation and purification of the tritridecylin employed has already been described elsewhere. The saponification number of this fat was 246·1 (calc. 247·3) and the setting-point 42·65°.

To each of the subjects V. and v. d. L. 25 g. of this fat completely melted up in thin broth or coffee together with a liberal amount of carbohydrate were administered, employing the standard technique. No tridecanedioic acid could be detected in the urine collected up to the next morning. In this case too, however, a considerable part of the fat supplied was excreted in the faeces in the form of large and small solid pieces.

In order to lower the melting-point below body temperature we next melted tritridecylin together with the same weight of coconut fat. From the experiments discussed in section (7) we knew that, after taking coconut fat, no substances are excreted in the urine which might interfere with the isolation in the pure state of any possibly excreted tridecanedioic acid. The subject V. partook in the usual way of 50 g. of this mixture, again together with a liberal amount of carbohydrate. No tridecanedioic acid was excreted in the urine.

Finally a mixture was made of 25 g. of tritridecylin with 10 g. of paraffin oil; such a mixture shows very little tendency to crystallise at body temperature. In the preceding experiment tritridecylin formed only 50 % of the weight of a fat mixture which could be completely adsorbed in the intestines; in the mixture just mentioned on the other hand tritridecylin is the only absorbable compound,

present in it to an amount of 70 %. It therefore appeared to us conceivable, and even not improbable, that on administering such a mixture tritridecylin would be absorbed at a greater rate than on administration of the mixture of tritridecylin and coconut fat, and consequently that there would be a greater chance of the accumulation of tridecanedioic acid in the blood and of the excretion of this substance in the urine. On the contrary, it is true that a reduction of the absorbability of tritridecylin as a result of the simultaneous presence of the paraffin oil may also be conceived. We believe on good grounds that any appreciable influence of the small amount of paraffin oil in this sense may be excluded; for example, it will be shown in a communication to be published later that the velocity of saponification of the simple triglycerides under the influence of pancreas lipase is not altered by the addition of paraffin oil. The subject V. partook of the mixture in question, completely melted up in thin broth together with a liberal amount of carbohydrate; no hindrance whatever due to the presence of the paraffin oil was experienced. Again no tridecanedioic acid appeared to be present in the urine collected up to the next morning.

Discussion.

(9) As appears from the preceding sections, administration under, as far as possible, corresponding conditions of different simple saturated triglycerides to one and the same subject leads to excretion of dioic (dicarboxylic) acid in the urine in very different amounts.

We only found a very considerable excretion of dioic acid after tricaprin and triundecylin had been taken. The amount of dioic acid excreted on the administration of always the same amount of the same triglyceride to one and the same subject under externally similar conditions can vary fairly considerably, while different subjects can also behave very differently with respect to the same triglyceride. We shall go into these facts, concerning which a few data are already to be found above, in later communications. It is consequently impossible to indicate yet on the basis of the results of the few experiments discussed above whether triundecylin or tricaprin has the greater tendency to cause dioic acid-acidosis and diaciduria; in other words—as we shall term it—which of the two is the more *diacidogenic*. Even after further experience obtained by us in the continuation of the work here described, we do not yet wish to state an opinion on this point. Trinonylin is certainly considerably less diacidogenic than the triglycerides just discussed, and in tricaprylin the diacidogenic properties are almost certainly still less developed. With the two higher triglycerides investigated by us, tritridecylin and trilaurin, dioic acid was either not found in the urine or at most only in traces, in spite of the selection of subjects with a considerable tendency to diaciduria.

A comparative review of the diacidogenic properties of the triglycerides investigated, based on the experiments described in this paper and therefore obviously being only of a preliminary nature, is given here.

	Term-number*	
Tritridecylin	12	Not diacidogenic
Trilaurin	11	Practically not diacidogenic
Triundecylin	10	Strongly diacidogenic
Tricaprin	9	Strongly diacidogenic
Trinonylin	8	Rather weakly diacidogenic
Tricaprylin	7	Weakly diacidogenic

* See Verkade and Coops [1930, 1].

The results of our experiments with tricaprylin justify the question whether the diacidogenic properties of the lower triglycerides only decrease slowly on descending the series and whether it will therefore, for example, be possible to prove the excretion of pimelic acid (heptanedioic acid) in the urine after the administration of triheptylin to suitable subjects. It did not at the time appear to us necessary to study this point.

On the other hand we can indeed say with certainty that with the triglycerides of higher term-number [Verkade and Coops, 1930, 1] than those we investigated (>12)—we leave out of consideration the difficulties which these substances would lead to on account of their high M.P.—there will be no question of dioic acid-acidosis or dioic acid excretion in the urine.

However puzzling this remarkable course of the diacidogenic properties may still be in the main, one thing appears very clearly, namely, that there is definitely no question of a contrast in this respect between the triglycerides derived from acids with odd and even numbers of C atoms; the behaviour of the triglycerides with the term-numbers 9 and 10, 11 and 12 may be compared.

In succeeding communications we shall show that the normal saturated fatty acids—we shall at present deal only with these—are catabolised in a hitherto absolutely unknown way besides the classical way of the β-oxidation discovered by Knoop, which from now onwards we shall call *one-sided β-oxidation*. By ω-oxidation of the fatty acid the corresponding dioic(dicarboxylic) acid is formed which is then further catabolised *via* lower dioic acids formed by *two-sided β-oxidation*. It may be remarked in passing that we in no way wish to exclude the possibility of the occurrence of still further mechanisms for the degradation of these fatty acids.

It is a fact that practically no one—and in our opinion certainly rightly—doubts the degradation of saturated fatty acids by one-sided β-oxidation. The arguments in favour of this way of degradation—one can hardly speak of proofs—are, however, really of little significance. The only indication in this direction with the fatty acids with even numbers of carbon atoms, the acid components of our normal food fats, really consists in the formation from them of ketonic bodies under various conditions, which need not be enumerated here, and with the fatty acids with odd numbers of carbon atoms in the non-occurrence of ketonic bodies under these same conditions. Finally the greatest support for the correctness of the view in question is derived from experience obtained with derivatives of the fatty acids, *e.g.* with the ω-phenyl-substituted fatty acids by Knoop, with the fatty acids containing a methyl(benzenesulphonyl)amino-group by Flaschenträger, *etc.* Closer investigation of this way of degradation of the fatty acids proper is quite definitely desirable; in fact such work is included in our programme.

On the other hand in the case of the way of degradation of the fatty acids now discovered by us we have been able to follow the degradation as it were from phase to phase both with the fatty acids with even and those with odd numbers of carbon atoms with the help of simple *in vivo* experiments. As far as the first phase is concerned, the ω-oxidation, the evidence for this statement is to be found in the experimental part of this paper.

(10) We shall naturally have to deal fully in later papers with the question of the factors to which is due the fact that administration of the various simple saturated triglycerides under as far as possible similar conditions to the same healthy subjects leads to accumulation of dioic acid in the blood and excretion of it in the urine in such typically divergent amounts. At present very little can be said with certainty on this subject. The catabolism of fat administered *per os*

takes place through a highly complicated series of processes, which we cannot as yet properly grasp at all even as regards its main features, and each phase of which will occur generally with a characteristic speed for each triglyceride, or each fatty acid, *etc.* Now it is certainly obvious to suppose that it will depend on the velocity relationships of all these processes whether, and if so to what extent, accumulation of dioic acid in the blood will occur after administration of a certain triglyceride and consequently—perhaps on exceeding a certain threshold concentration—excretion of this acid will take place in the urine. Experiments such as those described in this paper can—we already pointed this out expressly in section (2)—only serve for a first orientation with regard to the newly discovered way of degradation of the fatty acids and of the phenomena connected therewith. A deeper insight can only be obtained by comparative investigations of the behaviour of the triglycerides in question in different phases of the total process of fat metabolism. The first results of work carried out in this direction in our laboratory will soon be published.

(11) It may be stated here that by accepting the idea very briefly indicated above with regard to the cause of the characteristic serial change of the diacidogenic properties in successive triglycerides one may, or rather one must, logically conclude that there also exists some analogous change in the ketogenic properties of successive triglycerides, derived from fatty acids with even numbers of carbon atoms. Systematic work in this region has not yet been carried out. There is certainly also here a possibility of interesting and doubtless important investigations with the aid of perfectly defined synthetic fatty acid derivatives; we are meanwhile convinced that it will definitely be very difficult to reach really unambiguous results in this domain.

Summary.

1. Administration under as far as possible corresponding conditions of a series of simple saturated triglycerides to one and the same subject leads to excretion of dicarboxylic acid in the urine in very different amounts.

2. With respect to this ω-oxidation of the component fatty acid there is no contrast between triglycerides derived from acids with odd and even numbers of C atoms.

3. The excretion of dicarboxylic acid is largest with triundecylin and tricaprin; it diminishes on descending the series. No dicarboxylic acid is excreted after administration of the higher triglycerides.

4. The supposed cause of this peculiar serial change of the so-called diacidogenic properties of these successive triglycerides is indicated.

5. It is pointed out that consequently some serial change may or rather must exist in the ketogenic properties of successive triglycerides, derived from fatty acids with even numbers of carbon atoms.

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REFERENCES.

Baer and Blum (1908). *Hofmeisters Beitr.* **11**, 101.
van Eldik Thieme (1911). *Thesis, Delft*, p. 26.
Flaschenträger (1927). *Z. physiol. Chem.* **159**, 297.
Garner and Randall (1924). *J. Chem. Soc.* **125**, 881.
Jaeger (1917). *Z. anorg. Chem.* **101**, 1.
Loskit (1928). *Z. physikal. Chem.* **134**, 135.
Mori (1918). *J. Biol. Chem.* **35**, 341.
Scheij (1899). *Rec. Trav. Chim.* **18**, 193.
Taylor and Clarke (1927). *J. Amer. Chem. Soc.* **49**, 2829.
Verkade (1927). *Rec. Trav. Chim.* **46**, 137.
—— and Coops (1930, 1). *Rec. Trav. Chim.* **49**, 568.
—— —— (1930, 2). *Rec. Trav. Chim.* **49**, 578.
—— van der Lee and Meerburg (1932). *Rec. Trav. Chim.* **51**, 850.
—— —— (1933). *Proc. Kon. Akad. Wetensch. Amsterd.* **36**, 314.
—— Elzas, van der Lee, de Wolff, Verkade-Sandberg and van der Sande (1933). *Z. physiol. Chem.* **215**, 225.
Walker and Lumsden (1901). *J. Chem. Soc.* **79**, 1197.

VII. A NEW COLOUR TEST FOR THE DETERMINATION OF VITAMIN A.

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(*Received November 18th, 1933.*)

Since the paper of Carr and Price [1926] much attention has been directed towards the antimony trichloride reaction of vitamin A. Opinions as to the value of this test are not uniform; it is, however, generally emphasised that this colour reaction is not dependable enough for exact determinations; at best it can serve as an orientation within certain limits. In the determination of the vitamin A content of cod-liver oil this colour test yields uncertain readings. The colour of the reaction is not stable; at first the solution is blue but it soon becomes violet and finally red. For measuring the colour intensity of the Carr-Price reaction the Lovibond tintometer is generally adopted. Owing to the rapid colour change it is expedient to accomplish readings within a very short time—10 to 30 seconds. Moreover, the test is not specific for vitamin A, as other carotenoid substances show the same behaviour.

Owing to the drawbacks mentioned, several new tests have been proposed, none of which, however, is satisfactory.

We attempted therefore to devise a reaction characteristic of vitamin A and allowing its exact determination in cod-liver oil. We succeeded in finding an adequate test by a simple modification of the Carr-Price reaction: if the oil containing vitamin A is heated together with catechol and antimony trichloride, both dissolved in chloroform, the blue colour first produced soon turns into a violet-red. The following reagents are needed for the test:

1. Alcohol-free, absolute chloroform (lower alcohols and water impede the reaction).
2. 0·5 % solution of catechol in chloroform.
3. A cold saturated solution of antimony trichloride in chloroform.

The specimen of oil to be tested is first diluted with chloroform. To 1–2 ml. of this solution 1 ml. of the catechol solution and 2–3 ml. of the antimony trichloride solution are added. The test-tube containing the mixture is immediately transferred to a water-bath at 60° for 1–2 minutes. The blue colour first produced changes during this heating into an intense violet-red hue. In the cold this colour is not produced, as the change then takes the same course as without catechol. Subsequent heating of the solution which has undergone the change in the cold does not lead to the production of the violet-red colour; therefore heating must take place immediately after the reagents have been mixed.

Spectroscopic examination of the red fluid shows characteristic absorption bands. Four bands have been observed, two of which are pronounced enough to be measured with the diffraction spectroscope.

1. A broad band at the boundary of green and yellow, having indistinct limits; peak intensity at 552 $m\mu$.

2. A narrower, distinct band between green and blue; peak intensity at 476 $m\mu$.

Besides those mentioned, two more dim bands were observed, one in the blue and the other in the violet.

Solutions of vitamin A irradiated and thus inactivated with ultraviolet light do not give the reaction described; the solution becomes pink at once, and changes into brownish red with a bluish tinge.

Irradiation by means of X-rays has a similar effect: the solution first becomes red, then reddish brown.

As mentioned, the reaction of Carr and Price is given not solely by vitamin A, but also by other carotenoid bodies. By the courtesy of Professor Zechmeister, to whom our thanks are due, we have been able to examine carotene, lycopin, zeaxanthin, capsanthin and physalin. None of these carotenoids gave our reaction; they gave the original blue colour of the Carr-Price test and no change of colour could be observed on heating. We were unable to purchase other carotenoids, which will be studied later.

Thus our reaction makes it possible to differentiate vitamin A from carotene. Moreover, vitamin A can be demonstrated simultaneously with carotene; the red of vitamin A and the blue of carotene blend into a vivid violet.

The same reaction carried out with ergosterol shows the colour change in an inverse succession, the solution first becoming coloured pink which changes slowly into blue. No catechol is needed for this reaction; the colour change is, however, greatly hastened by its presence. Heating does not enhance it, moreover this is disadvantageous; when heated, the solution becomes green, but on cooling the blue colour reappears.

With irradiated ergosterol (vitamin D, vigantol oil) no colour reaction is observed.

The blue colour produced with ergosterol shows an absorption band between yellow and green, having a peak intensity at 595 $m\mu$; another band beginning at about 610 $m\mu$ and showing an increasing intensity towards the red can also be observed.

The catechol reaction of vitamin A is also suitable for quantitative determinations. Under certain conditions the intensity of the violet-red colour is proportional to the concentration of vitamin A and can be measured colorimetrically. A 0·01 % solution of potassium permanganate is used for comparison. A specimen of oil containing vitamin A is dissolved in chloroform, yielding a solution of a known concentration. 1 ml. of this solution is employed, or a smaller quantity is diluted with chloroform exactly to 1 ml. Now 1 ml. of the catechol solution and 3 ml. of the antimony trichloride solution are added and the test-tube is put into the water-bath at 60°. After the solution has become violet-red, it is compared in the colorimeter with the standard solution of potassium permanganate.

The correct performance of this reaction depends on the following conditions.

1. The duration of heating. Too short heating produces a red hue with a bluish tinge, whereas prolonged heating yields a brick-red solution.

2. The concentration of the catechol solution. The colour is different if the solution contains more or less than 0·5 % of catechol. More dilute solutions tend to produce a bluish red tinge, higher concentrations a brown one. Catechol solutions should always be freshly prepared, as solutions older than 24 hours are liable to give bluish tinges.

Of cod-liver oil from 0·1 to 0·5 ml. should be taken. When heated the violet-red colour appears in as short a time as 10 to 15 seconds. The reading

should be accomplished as quickly as possible, for within a short time the colour fades into brown. This change is probably caused by resinification of the oil; not more than 0·5 ml. of oil should therefore be used for the test.

Of vitamin A concentrates we examined two brands, called "Essogen" and "Vogan," respectively. Chloroform dilutions were prepared, which had to be heated for 1 to 2 minutes. As these solutions were examined in high dilutions, their oil contents were very low and consequently their colours lasted much longer, averaging 5 to 10 minutes and even more.

The following are the results of several determinations:

1. Cod-liver oil. (The comparator cell of the Duboscq colorimeter stood at the mark 10.)

0·1	ml. of oil	reading	18·0
0·2	,,	,,	8·9
0·3	,,	,,	6·2
0·4	,,	,,	4·6
0·5	,,	,,	3·6

2. 0·5 ml. of "Vogan" diluted to 10 ml.

0·1	ml. of solution	reading	10·0
0·2	,,	,,	5·0
0·3	,,	,,	3·2
0·4	,,	,,	2·4
0·5	,,	,,	2·0

3. 0·1 ml. of "Essogen" diluted to 10 ml.

0·1	ml. of solution	reading	30·0
0·2	,,	,,	15·2
0·3	,,	,,	10·0
0·4	,,	,,	7·3
0·5	,,	,,	6·0

The values above indicate that with an increase of the quantity of vitamin A the readings proportionally decrease, thus the colour intensity is a linear function of the concentration. Comparison of these values showed that "Vogan" contained 36 times, "Essogen" 60 times as much vitamin A as the cod-liver oil examined.

In another experiment we measured the absorption of our solutions photoelectrically. We employed a cell of 2·9 mm. in diameter. The maximum intensity of the photoelectric current amounted to $83{\cdot}5.10^{-7}$ amperes. 0·1 to 1·0 ml. of a 1 : 20 solution of Vogan was used. The results are shown in the following table:

ml. of solution	Current intensity 10^{-7} amp.	Absorption $83{\cdot}5/x$
0·1	47·5	1·8
0·2	39·5	2·1
0·3	28·5	2·9
0·4	22·5	3·7
0·5	18·0	4·6
0·6	13·5	6·2
0·7	10·5	7·9
0·8	7·0	11·8
0·9	5·5	15·2
1·0	2·5	33·4

Spectrophotometric examination of the colouring matter is being carried out. The relation of these data to the biological potency of the preparations examined is reserved for further study.

SUMMARY.

The reaction with catechol as described is an advance in two respects.

1. It makes possible differentiation of vitamin A from carotenoid substances.

2. The violet-red colour, similar to that of a dilute solution of potassium permanganate, is more stable than the blue colour obtained according to the Carr and Price method.

REFERENCE.

Carr and Price (1926). *Biochem. J.* **20**, 297.

VIII. POLARIMETRIC DETERMINATION OF GLUCONIC ACID.

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(*Received November 22nd, 1933.*)

The specific rotation of malic, tartaric and other α-hydroxy-acids is markedly increased by the formation of complex compounds with uranium salts and with molybdates, tungstates, *etc.* Use is made of this phenomenon in the estimation of optically active malates and tartrates in plant material [Auerbach and Kruger, 1923]. The writer is not aware that this phenomenon has been made use of in the estimation of gluconic acid. It can, however, be conveniently applied to this purpose.

Experimental.

Calcium gluconate recrystallised from water was dried at 100° and a 0·1 *N* (2·15 %) solution was prepared. 5 ml. samples of this stock solution were mixed with 2·0, 5·0, 10·0 and 15·0 ml. respectively of saturated ammonium molybdate solution; 2·0 ml. of glacial acetic acid were added, and each sample was made up to 25 ml. with distilled water. The rotations of these solutions were measured at various time intervals after mixing the reagents.

The results of nine typical experiments are given in Table I. It is found that the rotation of the gluconate-molybdate mixture is at its maximum value as soon after mixing of the reagents as it is possible to take readings (about 5 minutes). The rotation decreases during the first two hours to a value which is maintained almost unchanged for some days. It will be noted from Table I that the rotation of the gluconate-molybdate mixture attains its maximum value when 5 ml. of saturated ammonium molybdate are present in 25 ml. of solution. Trebling the concentration of molybdate only brings about a 3 % decrease in rotation, and halving the molybdate concentration correspondingly brings about a reduction in rotation of about 10 %.

Table I.

	ml. saturated molybdate per 25 ml. of solution	$[\alpha]_D^{20°}$ *		
		1 hour	3 hours	24 hours
I	2·0	343°	314°	—
II	2·0	331	—	—
III	5·0	372	361	361°
IV	5·0	373	—	366
V	5·0	371	—	—
VI	10·0	384	—	—
VII	10·0	362	352	—
VIII	15·0	362	350	—
IX	15·0	347	—	—

* Calculated with respect to quantity of total gluconic acid.

On the basis of these and other similar results, it was decided to treat gluconic acid or gluconate solutions in the following standard manner. 8 ml. of the gluconate solution are mixed with 5 ml. of saturated ammonium molybdate and 2 ml. of glacial acetic acid, and the mixture is allowed to stand for at least 3 hours in darkness. The rotation is then measured. Under these conditions, as the figures in Table II show, the relationship between gluconate content and rotation is almost linear when the gluconic acid content is less than 1·5 % and deviates only slightly at concentrations up to 3 %. In general, plant material containing gluconates or gluconic acid also contains carbohydrate. The specific rotation of glucose is not directly affected by treatment with the molybdate reagent, but relatively concentrated solutions (5 % for example) are fairly rapidly oxidised by the molybdate mixture, which itself becomes dark blue in colour. The rotation of the solution increases probably owing to the formation of gluconic acid among the products of oxidation of the glucose, and polarisation of the darkly coloured solution becomes impossible.

In the presence of sugars therefore the standard procedure mentioned above is varied. A measured volume of the solution is boiled with sufficient excess of NaOH to neutralise the acid and convert any gluconic lactone into sodium gluconate; calcium chloride is added and the solution treated with alcohol to make the alcohol content 80 %. The precipitated calcium salt is filtered, washed and redissolved in water so as to give 8 ml. of solution, to which 5 ml. of molybdate and 2 ml. of glacial acetic acid are then added. After standing 3 hours in the dark the solution is polarised as before.

Table II gives the observed rotations in a 2 dm. tube, and also the specific rotations when solutions of various gluconic acid contents were used in the standard manner described. The quantity of gluconic acid recorded in the left-hand column of the table is the quantity present in the 8 ml. sample used which is made up to 15 ml. by addition of molybdate and acetic acid. Sodium light was used in all cases and the temperature throughout was 22°.

Table II.

Quantity of gluconic acid, mg. in 8 ml.	Observed rotation in 2 dm. tube	Specific rotation
19·6	0·96°	367°
39·2	1·93	369
78·4	3·83	366
117·6	6·10	389
156·8	8·00	381

Intermediate values have been obtained by interpolation. It has been shown that the presence of citric acid does not affect the value of the specific rotation of gluconic acid in a manner comparable with the effect on malate-molybdate mixtures. An 8 ml. sample containing 78·4 mg. gluconic acid and 192 mg. citric acid in standard molybdate mixture gave a rotation of 3·96° in a 2 dm. tube. The specific rotation is therefore 378°.

Table III.

Acid	Specific rotation in presence of molybdate	Specific rotation in presence of uranyl salts
d-Malic	−720 to −770°	+510 to +525°
l-Malic	+720 to +770	−510 to −525
d-Gluconic	+366 to +390	About +80

The gluconate-containing material so far investigated in this laboratory has not contained malic or other optically active acids. It should, however, be possible to deal polarimetrically with such mixtures relatively easily on account of the different magnitudes (and directions) of the enhancements of rotation produced by molybdates and uranyl salts, of which data are given in Table III.

SUMMARY.

1. The specific rotation of *d*-gluconic acid is enhanced about 40-fold by formation of complex salts with molybdates. The value of the enhanced rotation is to a marked extent unaffected by variation in concentration both of gluconic acid and molybdate.

2. The rotations of gluconic acid complexes with uranyl salts and molybdates are in the same direction, in contrast to the behaviour of malates: hence polarimetric analysis of mixtures should be relatively simple.

REFERENCE.

Auerbach and Kruger (1923). *Z. Unters. Nahr. Genussm.* **46**, 97, 179.

IX. THE DETERMINATION OF BROMINE IN NORMAL BLOOD.

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From the Central Pathological Laboratory of the London County Mental Hospitals.

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During an investigation into the halogen constituents of the blood of psychotics, who, it is stated by Zondek and Bier [1932], have a greatly lowered blood-bromine, it was found that none of the methods available for blood-bromine determination was satisfactory. In particular that of Pincussen and Roman [1929], on which Zondek bases his conclusions, was shown to be subject to gross errors.

In all the methods the blood is first ashed with excess of alkali whereby the bromine is fixed as alkali bromide. The bromine is then liberated by oxidation with $KMnO_4$ and H_3PO_4 [Behr *et al.*, 1931] or HNO_3 [Pincussen and Roman, 1929] and collected in chloroform or carbon tetrachloride, or it is distilled over into potassium iodide solution. In any case it is difficult to avoid oxidising chloride and also to get rid of all traces of oxidising agents even if aeration is resorted to. In the method of Roman the melt is subjected to an alcoholic extraction which is claimed to remove potassium iodide only. The present author has shown, however, that although potassium bromide is only slightly soluble in alcohol the amount (100γ) present in the potassium carbonate melt from 10 ml. normal blood can be quantitatively extracted with 90–95 % alcohol, the bromide and iodide being thus extracted together. Fleischhacker and Scheiderer [1933] have shown that the oxidation with HNO_3 and H_2O_2 is not quantitative. In the Roman method excess of oxidising agent is removed by shaking the $CHCl_3$ layer with ice-cold NaCl solution. Hahn [1933] has shown that NaCl dissolves out some Br as well as the oxidising agents. In any case iodine titration in a water-chloroform mixture is a difficult procedure and not one capable of great accuracy.

According to van der Meulen [1931] bromides can be quantitatively oxidised to bromates by $2\frac{1}{2}$ times the theoretical amount of hypochlorite in the presence of borate buffer and half saturation with NaCl according to the equation

$$3KClO + KBr \rightleftharpoons KBrO_3 + 3KCl.$$

The completion of the reaction from left to right is ensured if the oxidation is carried out at 85° and excess hypochlorite is removed by addition of H_2O_2, which in turn is removed by boiling. Addition of bromates to iodides in acid solution results in the liberation of iodine equivalent to 6 times original amount of Br according to the equation

$$HBrO_3 + 6HI \rightleftharpoons HBr + 3H_2O + 3I_2.$$

It was found that the above procedure could be modified to deal with quantities of the order of 50γ such as are likely to be encountered in 10 ml. normal blood.

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It has the advantage that the oxidation and titration are carried out in the same vessel and all the oxidising agent is removed. A small amount of chlorate is unavoidably formed but this is allowed for by using controls which have been subjected to the same procedure from the start. Using this method of oxidising bromide and determining it by the iodine liberated it was found possible to evolve a method for determining bromine in 10 ml. normal blood.

Reagents required.

Water distilled from alkali in an all glass apparatus.

10*N* KOH and 10*N* K_2CO_3 purified so as to contain no bromide by von Fellenberg's [1926] method.

Pure ethyl alcohol distilled in all glass apparatus from KOH.

N KClO in *N*/10 KOH. Made by passing 71 g. chlorine gas, cooled and dried, into 1·8 litres of KOH containing 2·2 g. mols. or 123·2 g. KOH, finally making up to 2 litres with water. The KOH is contained in a flask cooled in ice while the chlorine is passing through to minimise formation of $KClO_3$.

N KI, preferably made fresh, contained in a brown glass bottle.

N HCl.

0·5 % starch; acid-washed soluble starch freshly made up each time required.

N H_2O_2.

N/500 sodium thiosulphate solution.

The proposed method for blood is as follows. 10 ml. of oxalated blood are measured into a 300 ml. flask. 0·8 ml. 10*N* KOH, 0·8 ml. 10*N* K_2CO_3 and 30 ml. water are added, and the whole is heated on the water-bath for 4 hours. The mixture is then transferred to a nickel basin of 10 cm. diameter and evaporated to dryness on the water-bath. The basin is dried at 150–160° for 60 mins. and then heated at 420° in the muffle furnace for 2 hours and finally at 500° for ½ hour. The contents are ground up with water by means of a glass rod, evaporated to dryness on the water-bath, dried at 150–160° for 30 mins., and then ignited for ½ hour at 500°. The grinding with water, drying at 160° and igniting at 500° for ½ hour are repeated. During ignition in the muffle the contents of the basin swell up considerably and there is danger of loss unless the surface of the melt is pricked with a finely pointed glass rod, thus allowing tarry vapours to escape. 20 ml. of water are then added to the basin, which is heated for a few minutes on the water-bath. The contents are ground up with a glass rod and then filtered through a 9 cm. Whatman's No. 5 filter-paper previously well washed, into a 9 cm. porcelain basin. The residue is repeatedly extracted with hot and finally cold water until there are about 50 ml. in the porcelain basin which are then evaporated on the water-bath. The porcelain basin is heated on the water-bath to dryness, carefully dried first at 120° for 15 mins., then at 150–160° for 15 mins., and finally ignited in the muffle for 10 mins. at 500°. The contents are dissolved in 15 ml. water and filtered through a 7 cm. Whatman's No. 42 filter-paper into another porcelain basin, the filter being washed several times with *N*/10 K_2CO_3. The solution is evaporated on the water-bath and the residue dried at 150–160° and ignited in the muffle for 5 mins. at 500°. It is then completely dissolved in 1·8 ml. water and alcohol added 5 ml. at a time with continuous stirring, the mixture being well rubbed with a round-ended glass rod, until 15 ml. alcohol have been added. The mixture is stirred until the K_2CO_3 passes into a pasty condition when the alcoholic layer is decanted through a Jena glass sintered filter No. 3 into a 30 or 40 ml. flask. The residue is extracted with two more successive lots of 5 ml. of alcohol. To the potassium carbonate from which the alcohol has been drained

is then added 0·6 ml. water and the above alcoholic extraction is repeated. The contents of the flask are evaporated on the water-bath to dryness, with addition of a couple of pumice granules to facilitate smooth boiling and, if the flask looks very full, under a stream of nitrogen. The last traces of alcohol are removed by heating at 150–160° for 10 mins. To the residue are added 2 ml. water, 1 ml. KClO and 2·5 ml. saturated NaCl, and the mixture is warmed to about 85° on the water-bath and treated with 1 ml. saturated H_3BO_3. The flask is removed after 20 mins. and cooled and then 1 ml. N H_2O_2 added, after which the contents are gently boiled for 5 mins. The flask is cooled and 1·5 ml. N HCl, 5 drops of starch and 1 ml. N KI are added, and the solution is titrated against $N/500$ thiosulphate using a 3 or 5 ml. micro-burette

$$1 \text{ ml. } N/500 = 26{\cdot}7\gamma \text{ bromine.}$$

The oxidation of the bromide in the presence of chloride is very conveniently carried out in the same flask as the titration so that there is very little chance of loss.

The preliminary heating on the water-bath with water and alkali causes partial if not nearly complete hydrolysis of the blood-proteins, and as the author has reason to think some of the bromine in the blood is bound to protein, there is less likelihood of loss and at the same time the use of huge quantities of alkali with consequent corrosion of dishes and basins is avoided. It also permits the use of nickel and porcelain basins for the ashing.

The chief aim in the ashing process is to evaporate most of the tarry matter at 400° and then to reduce the ash so that there is very little carbon left by heating at 500°. Grinding up the ash with water and igniting three successive times at 500° seems to remove any possibility of organic matter coming through to the hypochlorite stage. If organic matter is present it will lead to low figures. The method of ashing will not recover substances like bromobenzene or ethyl bromide which are immiscible and evaporate before fixation, but there is no reason to believe that such substances exist in blood.

The contents of the flask after the addition of the KClO must not be boiled as there is danger of excessive $KClO_3$ formation. The titration end-point is quite sharp, but owing to the large salt content of the solution the colour change is from blue-purple to colourless. The blank determination on the 10 ml. sucrose solution control which is put through exactly the same process is of the order of 0·4 ml. of $N/500$ thiosulphate and is due to a small amount of chlorate forming during the heating at 85° on the water-bath and includes the inevitable over-titration so that these both cancel out.

Iodides are also quantitatively oxidised by hypochlorite to iodates and are thus estimated together with bromide.

Normal blood, however, contains 500–1000γ/100 ml. bromine and 10–15γ/100 ml. iodine, and thus variations in the latter unless very large, as for instance after iodine therapy, do not appreciably affect the figures for bromine.

Table I. *Showing amounts of bromine as γ Br found in 10 ml. samples of normal human blood together with amounts found after bromide had been added.*

	Normal blood	+100 γ Br as KBr	Difference
A	89	184	95
B	120	217	97
C	173	277	104

SUMMARY.

A method is described for the determination of the bromine content of 10 ml. of normal blood, whether it contains 0·1 mg. or 100 mg./100 ml.

Through the courtesy of Sir Robert Robertson and the late Sir Walter Fletcher the author has had access to an unpublished report of work on the determination of iodine carried out for the Medical Research Council at the Government Laboratory by Mr C. O. Harvey, and he has been guided by it in choosing the conditions for the preliminary treatment and incineration of the blood and for the alcoholic extraction of the potassium bromide. He wishes to express grateful thanks for this privilege.

The author also wishes to thank Dr F. L. Golla, Director of the Central Pathological Laboratory, and Dr S. A. Mann for their advice and criticism. He is also indebted to the medical officers of the London County Mental Hospitals for their kindness in providing the samples.

REFERENCES.

Behr, Palmer and Clarke (1931). *J. Biol. Chem.* **86**, 316.
von Fellenberg (1926). *Ergebn. Physiol.* **25**, 176.
Fleischhacker and Scheiderer (1933). *Klin. Woch.* **12**, 391.
Hahn (1933). *Klin. Woch.* **12**, 390.
van der Meulen (1931). *Chem. Weekblad.* **28**, 82.
Pincussen and Roman (1929). *Biochem. Z.* **207**, 416.
Zondek and Bier (1932). *Klin. Woch.* **1**, 633.

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X. OBSERVATIONS ON THE ACTION OF CHLORINE ON LIGNIFIED TISSUES.

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During a study of the chemical nature of the cell membrane in plant tissues use was made of the chloramine reaction as a means of detecting protein in the cell-wall. Plant tissue sections were submitted to the prolonged action of chlorine gas and subsequently treated with aqueous solutions of sodium hydrogen phosphate and potassium iodide. Under these conditions proteins, if present, produce chloramines which liberate iodine from potassium iodide, resulting in a yellow coloration of the cell-wall [Wood, 1926].

This reaction led to interesting results with lignified tissues apart from its original application. A pink coloration was developed in these tissues [Wood, 1927], and while investigating the origin of this it was found necessary to devise some means of detecting vanillin in the cell-wall and of distinguishing this compound from coniferin, since it was thought possible that the coloration might be due to a compound of vanillin or coniferin, or their derivatives, with chlorine. Other possibilities were that it might be due to a compound of lignin with chlorine or to the nature of the lignin present. It has been shown by the author that this coloration is not due to oxidation of the cellulose base, but to the presence of the halogen [Wood, 1927].

Vanillin has been shown to occur in linden bark, in the summer only, by Bräutigam [1900, 1, 2]; it was found in a board by von Lippmann [1904], is stated to predominate in the oldest lignified tissues by Ellram [1896] and has been shown to occur in decayed oak-wood by Sullivan [1914].

It is well known that the sulphite and soda liquor from the manufacture of wood-pulp contains vanillin.

Coniferin occurs frequently in plant material. Kubel [1866] found it in the cambial sap of all conifers. It is converted by hydrolysis into coniferyl alcohol, and by subsequent or direct oxidation into vanillin [Tiemann and Harmann, 1874]. Hence it was necessary to perform distinguishing tests for these substances.

The possibility of the production of vanillin from coniferin, or on further oxidation vanillic acid by the chlorine-phosphate-iodide treatment to which the plant sections were submitted, must be taken into account, for Tiemann and Mendelsohn [1875] record the reverse process.

Should vanillin be detected after chlorination, its origin is a matter of some importance.

As the pink coloration only appeared in the presence of the halogen, and evidently a definite compound was formed, the problem was to show whether it was due to:

(*a*) a compound of chlorine with coniferin, or coniferyl alcohol which may be produced by the hydrolysis of coniferin;

(*b*) oxidation of coniferin to vanillin and the subsequent formation of a chlorovanillin;

(*c*) the formation of chlorovanillin from vanillin naturally present in the cell wall;

(*d*) the prolonged treatment with chlorine resulting in the formation of vanillic acid or possibly the production of a chlorovanillic acid.

No information could be obtained as to the existence of any compound of coniferin or coniferyl alcohol with chlorine, so that this part of the work was concerned with proving the presence or absence of the coniferyl nucleus before and after chlorination.

Menke and Bentley [1898] describe the formation of a chlorovanillin when vanillin in solution in chloroform is treated with chlorine for some time. Hann [1925] records the preparation of chlorovanillin, probably identical with the above, when vanillin dissolved in glacial acetic acid is treated with chlorine in the presence of fused sodium acetate.

But these chlorovanillins are colourless or yellow substances and the compound under investigation is pink, hence if chlorovanillin is produced, there must be some further reaction, resulting in a coloured compound.

Hann *et al.* [1929] describe the preparation of 5-chlorovanillal-3-nitro-*p*-toluidine in the form of iridescent brick-red granules. It should be noted that this possesses a deep colour because it is a nitro-anil; the colour is not associated with the presence of the chlorine.

They prepared also iodo-derivatives of substituted vanillins with the isomeric tolylrhodanic acids, and found that as a general rule all the iodo-derivatives are red, brown or deep yellow, whereas the analogous chlorine compounds are yellow. All these compounds were found to be insoluble in water, slightly soluble in alcohol and organic solvents, and to dissolve in concentrated sulphuric acid with the production of brilliant red colours. Hence there are two further possibilities of the origin of the observed pink colour; *viz.* it may be due to:

(*a*) the production of chlorovanillin and its subsequent union with an amine derived from the protoplasmic constituents of the cell, or from the cell-sap, to produce a chlorovanillal derivative which then reacts with the potassium iodide;

(*b*) to the formation of an iodo-derivative of the substituted vanillin on treatment with potassium iodide.

Experimental.

The experimental work was carried out on plant tissues, in the form of transverse sections of stems, and where possible similar experiments were performed on the chemical substances suspected. The twenty-three species examined included those which had been found already to give an intense pink coloration, those which gave a faint coloration and some which gave negative results. They included Gymnosperms, which all gave an intense coloration, and Angiosperms which gave a variety of results.

Owing to the comparatively small proportion of any section of the plant tissue giving the pink coloration in the chloramine reaction, no method has been devised so far for the extraction of the substance responsible, hence most of the work had to be based on colour reactions, although wherever possible these represented a definite chemical reaction and were not unexplained colour effects.

When bromine is used instead of chlorine a similarly coloured body is produced, though this element is not to be preferred to chlorine for use in this test [Wood, 1931].

The remote possibility of the metallic ion used having some effect also had to be considered.

A similar coloration to that produced when potassium iodide was used was obtained with sodium iodide and with magnesium iodide, it was therefore inferred that the nature of the metallic ion was without influence on the coloration. With potassium chloride, no pink coloration was obtained, and with potassium bromide, an orange-red coloration, which did not resemble the usual pink colour associated with this test, but was somewhat like that obtained when bromine was used instead of chlorine.

These results appear to give additional proof that the effect is connected with the presence of iodine.

In practice all results obtained were more satisfactory if the material, which was kept in alcohol, were placed in water for 12 to 24 hours before being submitted to the action of chlorine. The chlorinated material was then kept in alcohol (as otherwise, in spite of the presence of chlorine, fungi sometimes attack the sections) being treated with water again before completing the test.

Certain products of the chlorination of lignin dissolve in alcohol, *e.g.* lignone chloride [Cross and Bevan, 1895]. Although this substance is light yellow in colour, it was thought possible that it might react with iodine to produce a substance of a different colour. By the use of alcohol the lignone chloride is partially removed before proceeding with the rest of the test.

Schorger [1926] states that the yellow colour produced in lignified tissues by the action of chlorine is due to a combination of the chlorine with the lignin, and that if a dilute solution of sodium sulphite is applied, as in the Cross and Bevan method of determining cellulose, the liquid is coloured pink to purple-red [Bevan and Cross, 1880; Webster, 1883]. By this method the chlorinated products are stated to be removed from wood. Hence the next step was to remove such products of chlorination from the sections. This was effected by boiling them with 2 % sodium sulphite solution after chlorination, then treating them with sodium hydrogen phosphate and potassium iodide and comparing the colour reactions with those produced in sections which had not received the sodium sulphite treatment.

Transverse sections of the stems of fifteen species were examined in this way, chlorination being effected by chlorine gas and by chlorine dissolved in carbon tetrachloride. The pink colour appeared in the same way as if sodium sulphite had not been used, and there was only extremely slight coloration of the liquid.

Sections extracted with glacial acetic acid, which also dissolves lignone chloride, failed to give the test.

The last two series of experiments also show that the more satisfactory results obtained when material that has been preserved in alcohol is treated with water before and after chlorination should not be ascribed to the partial removal of lignone chloride, since the presence of this substance apparently has no effect upon the development of this pink coloration, although a certain amount of this substance is probably formed.

It was next necessary to show whether the pink colour was associated with the lignin complex or not; in investigating this point a large number of plants were examined. The reactions employed for lignin were those produced with diamine blue, brilliant Congo blue, orcinol, phloroglucinol, *o*-, *m*- and *p*-xylidene, and *m*-xylidene acetate.

In the majority of these reactions the pink colour was found to be associated with a positive reaction for lignin in primary and secondary xylem, and in many

cases in stelar sclerenchyma. The results obtained with cortical and stelar sclerenchyma were indefinite, pointing to considerable variation in the chemical composition of these tissues.

Since a connection had been established between the reaction and the lignin complex, the next step was to determine what parts or products of this complex were concerned. It was evident from the amount of the coloration, which in no case was so intense as to render the cell-wall opaque to light, that a portion only of the bulk of the cell-wall was concerned.

There are two types of lignin described by Paloheimo [1929], *ortho*lignin, which is resistant to the action of concentrated hydrochloric acid and is scarcely attacked by boiling with dilute alkali, and other kinds of lignin, called hydrolysable lignins, which are readily removed by strong acids and dilute alkalis.

Sections of the stems of seven species were treated separately with caustic soda and with hydrochloric acid and the aniline sulphate and Maule tests used to give the characteristic lignin reactions. The results were compared with those obtained from similar tests made on untreated material, the depth of colour indicating how much lignin had been removed.

All the above tests were performed both before and after chlorination and correlated with the chloramine test made on other sections of the same material. The pink colour is not influenced to any marked degree by the presence or absence of either *ortho*lignin or hydrolysable lignin.

The next possibility seemed to be that the colour was due to some compound either produced in small quantity from the lignin as a whole, or loosely associated with the lignin, or perhaps to some encrusting substance. As indicated earlier, two such substances are coniferin and vanillin. Differential experiments were made for the detection of coniferin and vanillin in plant-tissues, and the results obtained were correlated with those of similar experiments made with vanillin and coniferin themselves. These compounds were submitted to the action of chlorine gas, or to that of chlorine dissolved in carbon tetrachloride, and in every case to subsequent treatment with sodium hydrogen phosphate and potassium iodide. The colorations produced were mainly yellow to orange or brown with coniferin, and orange, red or reddish brown with vanillin, indicating that the latter was more likely to be associated with the coloration produced in plant tissues.

In general, the xylem formed in the autumn gave a stronger vanillin reaction than the spring xylem, which often indicated traces of coniferin. The older lignified tissues gave a stronger reaction for vanillin than the younger tissues, thus confirming Ellram's work [1896], and the younger tissues sometimes gave only a very slight reaction. This suggests that vanillin may be produced from coniferin as the tissues become older. Where there was a positive chloramine reaction, the colour reactions for vanillin were always more intense, particularly in two of the species examined, *viz. Laurus nobilis* and *Bambusa nigra*, and where there was a negative chloramine result, the reactions for vanillin were either negative or extremely slight.

As might be expected from the earlier work the reactions varied with the sclerenchymatous tissue involved [Wood, 1927]. The variation was mainly in the shade of the coloration indicating either vanillin or coniferin, the general result being that vanillin in varying amounts was indicated. The reactions for vanillin were generally stronger in cortical sclerenchyma than in stelar sclerenchyma.

The results obtained with all these tissues show that vanillin, and not coniferin, has some connection with the pink colour of the chloramine reaction.

This eliminates the possibility of it being due to a compound of chlorine with coniferin or coniferyl alcohol.

Since it was possible that coniferin, present in the cell-contents, or the cell-wall, might have undergone oxidation by the prolonged chlorine treatment, ultimately producing vanillin, the results of the previous experiments on material, both before and after chlorination, were carefully examined. Sections of six species were again submitted to the codeine test for vanillin [van Itallie and Steenhauer, 1927, 1, 2] both before and after chlorination. In most cases the vanillin appeared to be present before as well as after chlorination, showing that it was naturally present in the material and was not an oxidation product of chlorination.

The possibility of some of the reactions attributed to coniferin and vanillin being also given by hemicelluloses could not be ignored, particularly as these substances may be transition products in the change from pectic substance to lignin, when lignification of the cell-wall takes place [O'Dwyer, 1928].

Schultze defines hemicelluloses as the carbohydrates occurring in the plant-cell that are dissolved by dilute alkalis, readily hydrolysed by hot, dilute mineral acids and destroyed by Schulze's reagent [Schulze, 1890; 1891; 1892]. Schorger [1926] in addition considers that they should be soluble in boiling water.

Hemicelluloses, such as wood gum or xylan, may be present in the cell wall. In order to determine whether wood gum or xylan were present in the original material used, the following experiments were carried out with sections of stems of eight species. Some sections were treated with 5 % aqueous caustic soda in the cold and the usual phloroglucinol test applied, both in the cold and on warming.

The latter test was also made on untreated material both in the cold and on warming. The xylan is removed by caustic soda. Xylan is coloured by phloroglucinol on warming, while lignin is coloured in the cold [Wheeler and Tollens, 1889]. If a positive reaction is produced on the untreated material in the cold this is due to lignin alone, but the coloration produced on warming is due to the combined action of lignin and xylan. Naturally if xylan is removed with caustic soda, any coloration is due to lignin alone. Maximum effect was produced if the sections, which had been treated with caustic soda, were left in water for some days before being treated with phloroglucinol.

The colour produced with phloroglucinol was even more brilliant when the sections were treated with caustic soda, which seems to show that in these specimens there was not any appreciable amount of xylan and certainly not enough to have any marked effect in the tests with which this paper is concerned.

The colour produced with phloroglucinol was examined carefully in every case for any reddish-violet tinge, as this indicates the presence of a compound of *iso*-eugenol with oxygen [Herzog and Hillmer, 1929]. Dilute alkalis produce *iso*-eugenol from eugenol, and this on oxidation gives vanillin.

Only in two of the Gymnosperms, *Cycas revoluta* and *Larix europaea* was any purplish tinge perceptible in untreated material in the cold and on warming. In *Larix europaea* when the material had been treated with caustic soda this coloration persisted, but only when the phloroglucinol test was made in the cold. This shows that *iso*-eugenol was not usually present in the material used in these tests.

Caustic soda removes any coniferyl alcohol present, so that none of the above results, after soda treatment, can be attributed to this substance.

The possibility of the connection of vanillin and chlorine with the development of the pink colour obtained in the chloramine reaction having been

established the nature of the compound present was next investigated. Further confirmation of the responsibility of the halogen was afforded by the analogous results which were obtained when bromine was substituted for chlorine [Wood, 1931]. Chlorovanillin, at first sight, appears to be an obvious solution, but this substance is colourless or light yellow in colour, so, should the suspected connection be true, the compound responsible for the coloration must be a product of the reaction of chlorovanillin with either (*a*) the other reagents used, (*b*) some substance occurring in the cell or cell-wall, or (*c*) with both the former and the latter.

Chlorovanillin was prepared according to Hann's method [1925], and experiments were made with it in the endeavour to clear up these points. In the first place, the reagents used, *viz.* sodium hydrogen phosphate and potassium iodide, were investigated.

Chlorovanillin was used (*a*) dissolved in alcohol, (*b*) in hot water, alone and in the presence of acetic acid, and separate experiments were carried out with each of these four solutions, using:

(1) sodium hydrogen phosphate and potassium iodide;
(2) sodium hydrogen phosphate;
(3) potassium iodide in which iodine had been dissolved, to obtain the maximum effect of the iodine which may be set free by the chloramine produced from any protein present in the cell-contents.

In no case was there any indication of a pink coloration, hence chlorovanillin and the reagents used are not alone responsible for this pink colour.

Since 5-chlorovanillin can form vanillal derivatives with amines (see above), the next experiments involved the examination of the effect of sodium hydrogen phosphate and potassium iodide upon:

(*a*) chlorovanillin and an amine;
(*b*) vanillin and a chloramine;
(*c*) chlorovanillin and a chloramine.

The slight acidity due to the sodium hydrogen phosphate must be taken into account when considering the results obtained, hence a similar series of experiments was conducted in which the phosphate was omitted.

Further, since the possibility of the effect being due to a substituted compound of vanillin had to be considered, the whole of this part of the work was repeated substituting vanillic acid for vanillin and vanillic acid treated with chlorine for chlorovanillin.

The experiments were conducted using:

(*a*) sodium hydrogen phosphate and potassium iodide;
(*b*) sodium hydrogen phosphate alone;
(*c*) potassium iodide alone, on all the substances enumerated above. All the solutions used were alcoholic, ethyl alcohol being employed. Sodium hydrogen phosphate and potassium iodide were used in the solid form.

The compounds containing chlorine and an amine group used were chloramine-T, *p*-chloroaniline, *o*-chloroaniline, and the compounds containing the amine group alone were dimethylaniline, dimethylaniline hydrochloride, aniline hydrochloride and xylidine acetate. In the first series of experiments the method of procedure was to add the amine to the vanillin in a test-tube, boil and then to add a little solid sodium hydrogen phosphate and potassium iodide, then a few drops of water, just sufficient to effect the solution of these substances, and to boil again. The resulting liquid was then put into small porcelain dishes and a

strip of filter-paper immersed in it, with one end out of the liquid, to observe the effect of the mixture upon cellulose, the object being to simulate an encrusting substance upon a cellulose base as might occur in the plant cell-wall.

Slow evaporation to dryness in the air was allowed to take place and the effect on the filter-paper carefully watched. As one end of the filter-paper was arranged to be out of the liquid, the colour effect produced could be observed as the liquid crept up the paper and then slowly evaporated. By arranging the amount of liquid in each dish carefully, the free end of the filter-paper was kept dry at the top. This was necessary, as many of the colour effects produced appeared as the paper dried just where the wet paper met the dry paper. The second and third series of experiments were conducted in a similar way, except that in the second series the potassium iodide was omitted, and in the third series no sodium hydrogen phosphate was used.

The three series of experiments were repeated using a strip of superfine "Tarantulle" instead of the filter-paper.

Finally, the whole of the experiments were repeated, as a confirmation of the results obtained.

In the latter series of experiments in addition to the filter-paper and superfine "Tarantulle", strips of purified wood-pulp were used.

Vanillin produced a pink colour on these materials when used with *p*-chloroaniline and *o*-chloroaniline in the presence of potassium iodide, the most brilliant effect being produced when both the sodium hydrogen phosphate and the potassium iodide were employed. Chlorovanillin produced a similar pink colour with *p*-chloroaniline and *o*-chloroaniline when potassium iodide was present alone and also when sodium hydrogen phosphate was used as well.

Vanillic acid gave a slight pink coloration in the presence of *o*- and *p*-chloroaniline respectively when the iodide was present. It is possible that this may be due to minute traces of vanillin in the vanillic acid.

Vanillic acid treated with chlorine gave indefinite results, owing to the liberation of iodine, which obscured any other colorations that might have occurred.

There was some indication of a faint pink coloration with both *o*- and *p*-chloroaniline, possibly due as indicated above to minute traces of vanillin.

In the last series of experiments it was found that frequently a considerable time had to elapse, often as much as twelve hours, before the colour developed, so that in every case the strips of material were examined the following day, in addition to the preliminary examination. This was probably due partly to variation in the atmospheric saturation and partly to variations in temperature which altered the rate at which evaporation of the liquid took place. This evaporation could not be increased by gentle warming as then either the colour did not appear at all, or it appeared only momentarily and rapidly disappeared.

In no case was it a permanent coloration, fading even at the ordinary temperature in the course of a few days.

Similar effects have been observed in sections of plant material which developed a pink coloration in the xylem after being submitted to the chloramine test which has already been described. In these cases also this coloration takes a certain time to develop and fades in the course of a few hours.

As additional proof that chlorine is an element essential to the reaction, and as a control to the other work, another series of experiments was made, using strips of filter-paper, "Tarantulle" and wood-pulp as before, and employing vanillin and vanillic acid respectively, with dimethylaniline hydrochloride and aniline hydrochloride and so having no chlorine present either substituted in the

vanillin nucleus or in the amino compound. These experiments were entirely negative.

Hence the results indicate that:

(1) potassium iodide (*i.e.* iodine) is essential to the reaction;

(2) the presence of sodium hydrogen phosphate is without effect upon the reaction;

(3) an amine and chlorine must be present.

The fact that an amine is essential to produce this coloration eliminates the possibility of its being due to an iodo-derivative of the substituted vanillin.

The conclusion that must be drawn is that in these experiments most probably some kind of vanillal derivative is produced from the vanillin, chlorine and amine, similar to those prepared by Hann *et al.* [1929] and that it reacts with the iodine to produce a pink-coloured substance, though whether the compound is an additive or substituted one remains to be determined. The method of experiment introduces many variables including atmospheric oxidation of the amines, but it was employed because it was to similar conditions that plant sections were exposed during the experiments made upon them.

To elucidate the reactions that take place in plant material is a difficult matter, but with reference to the pink coloration produced in the chloramine reaction when employed with such material the following facts have been proved:

(*a*) it is associated with vanillin and chlorine;

(*b*) it only appears when iodine is subsequently present.

Although the cell-wall does not contain any appreciable amount of protein [Wood, 1926], its close association with the cell-contents, which are rich in amino-compounds, renders these available for possible reactions. The experiments with chemical substances indicate that colorations are produced, when the conditions are such that a vanillal derivative might be formed, which, reacting with iodine, produces a substance resembling in colour and properties that appearing in the lignified tissues of some plants when treated with chlorine and subsequently sodium hydrogen phosphate and potassium iodide.

The inference drawn is that most probably in this reaction chlorine acts upon the vanillin in the cell-wall, forming chlorovanillin which reacts with a chloramine or mixture of chloramines, produced from protein within the cell to produce a vanillal, which gives a coloured substance when iodine is present.

Should the chlorine not affect the vanillin to any marked degree, probably vanillin itself may react with the chloramines from the cell-contents and still produce the coloration.

It is possible that both series of reactions take place simultaneously.

Having found some indication of the kind of substance producing this coloration, and the chemical experiments with filter-paper, "Tarantulle" and wood-pulp showing that it is possible that the vanillin responsible is absorbed or at least very loosely united with some form of lignin as a base, experiments are now being carried out to investigate these points and the chemical nature of the base.

Since it has been found that there is more vanillin in older than in younger tissues, and more in autumn than in spring xylem, the author suggests that this substance may be present as an excretory product, which is set free when the function of the tissues becomes more largely that of mechanical support. This upholds the theory that has been advanced that the vanillin present is either adsorbed upon the lignin base or only loosely associated with this base.

SUMMARY.

1. When investigating the origin of a pink colour produced in lignified tissues when the chloramine reaction is employed, differential tests for coniferin and vanillin were made.

2. Experimental work is described in which it is shown that the coloration is not due to lignone chloride or to the products of chlorination removable by sodium sulphite, that it is closely associated with the presence of iodine and also of lignin, but that the nature of the lignin itself is not concerned, so that the latter association is either very loose or the substance is encrusted on the lignin. It is shown that vanillin is present when the coloration is produced, and that there is more vanillin in older than younger tissues, in autumn than spring wood, in cortical than in stelar sclerenchyma, and that it is naturally present in the cell-wall. Hemicelluloses and coniferyl alcohol are not responsible for the observed effect.

3. Under similar conditions to those used with plant sections experiments were made with vanillin, chlorovanillin, vanillic acid, vanillic acid treated with chlorine, and amines or chloramines, in which filter-paper, "Tarantulle" and wood-pulp were used upon which to "encrust" the compounds concerned. Indications were obtained that a vanillal derivative was formed which reacted with iodine to produce a similarly coloured substance to that obtained in plant tissues.

4. The conclusion drawn is that vanillin, in loose association with some form of lignocellulose, is acted upon by chlorine producing a chlorovanillin, which with the chloramines produced from protein within the cell, forms a vanillal derivative which gives a pink-coloured substance in the presence of iodine.

5. The possibility that vanillin is an excretory product, produced when the function of the tissues concerned has become chiefly that of mechanical support, is discussed. This theory is upheld by the fact that vanillin predominates in older tissues and in autumn wood.

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REFERENCES.

Bevan and Cross (1880). *Chemical News*, **42**, 80.
Bräutigam (1900, 1). *Pharm. Ztg.* **45**, 164.
—— (1900, 2). *Arch. Pharm.* **238**, 556.
Cross and Bevan (1895). Cellulose, 136. (Longmans, Green and Co., London.)
Ellram (1896). *Chem. Centr.* **2**, 99.
Hann (1925). *J. Amer. Chem. Soc.* **47**, 1998.
—— Jamieson and Reid (1929). *J. Amer. Chem. Soc.* **51**, 2586.
Herzog and Hillmer (with Paersch and Hellriegel) (1929). *Ber. deutsch. chem. Ges.* **62**, 1600.
Kubel (1866). *J. prakt. Chem.* **97**, 243.
Menke and Bentley (1898). *J. Amer. Chem. Soc.* **20**, 316.
O'Dwyer (1928). *Biochem. J.* **22**, 381.

Paloheimo (1929). *Biochem. Z.* **214**, 161.
Schorger (1926). The chemistry of cellulose and wood, 123, and 141. (McGraw-Hill Book Co., New York.)
Schulze (1890). *Z. physiol. Chem.* **14**, 227.
—— (1891). *Ber. deutsch. chem. Ges.* **24**, 2285.
—— (1892). *Z. physiol. Chem.* **16**, 391.
Sullivan (1914). *J. Ind. Eng. Chem.* **6**, 919.
Tiemann and Harmann (1874). *Ber. deutsch. chem. Ges.* **7**, 608.
—— and Mendelsohn (1875). *Ber. deutsch. chem. Ges.* **8**, 1136.
van Itallie and Steenhauer (1927, 1). *Arch. Pharm.* **265**, 696.
—— —— (1927, 2). *Pharm. Weekblad.* **64**, 925.
von Lippmann (1904). *Ber. deutsch. chem. Ges.* **37**, 4521.
Webster (1883). *J. Chem. Soc.* **43**, 25.
Wheeler and Tollens (1889). *Liebig's Ann.* **254**, 332.
Wood (1926). *Ann. Bot.* **40**, 547.
—— (1927). *Ann. Bot.* **41**, 281.
—— (1931). *Ann. Bot.* **45**, 421.

XI. URICASE AND ITS ACTION.
VII. SOLUBLE URICASE.

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It has been shown in Parts II, III and IV [Truszkowski, 1930] of this series that tissue uricase is associated exclusively with the insoluble fraction of the tissues, and that it is not extractable by aqueous solutions of p_H 6–11, by aqueous glycerol or by other agents generally used for extraction of enzymes.

Ro [1931] described a method of extracting uricase from tissues similar in principle to that of Wiechowski and Wiener [1907], whereby he obtained extremely active extracts. Ro's method consists in the acetone precipitation of an aqueous extract of ox-kidney followed by repeated treatment of the precipitate with acetone, alcohol and ether. The white powder thus obtained yields uricolytic solutions on extraction during an unspecified time with 0·125 % Na_2CO_3 solution.

It was found by the present author that the dry preparation prepared in the above manner yielded active filtrates after extraction during 24 or 48 hrs. at 37° with Na_2CO_3.

The possibility of obtaining soluble uricase by Ro's method having thus been confirmed, the necessity arose of reconciling this finding with the negative results previously obtained. The differences between the two methods consisted in the use of dry, lipoid-free kidney tissue by Ro, in the presence of water-soluble kidney constituents, and in the use of Na_2CO_3 solution for extraction, instead of the glycine-NaOH buffers used by the present author. With regard to the first point, it seemed improbable that the extractability of uricase could be affected by merely drying the tissues; the necessity of the alcohol-ether treatment should lie rather in removal from the tissues of some substance, probably lipoid, which prevents extraction from fresh tissues. As to the second point, it has been shown (Part IV) that the water-soluble constituents are inactive in the fresh state; experiments described below show that the same applies to this fraction after treatment according to Ro's method. Finally, extractions performed on fresh kidney tissue with aqueous Na_2CO_3 were found to yield inactive filtrates.

Experimental.

Ox-kidney uricase was prepared by the method described in Part IV of this series. Uric acid was dissolved in boiling aqueous NaOH (65 ml. 0·1*N* NaOH per g. uric acid); the solutions so prepared had p_H 7·5–7·8. Uric acid was determined by the method of Folin and Denis.

1. *Conditions of extraction.*

Since Ro's paper gives no details as to the duration and temperature of extraction, the first step was to determine these conditions. 1 g. of dry product prepared according to his method was suspended in 100 ml. of 0·125 % Na_2CO_3,

1 ml. of toluene was added to ensure sterility, and the activity of the extract was determined after standing 1, 24 and 48 hrs. at 35° by adding 5 ml. of filtered extract to 10 ml. of 0·144 % sodium urate, with 1 ml. of toluene, and determining uric acid after incubation at 35° during 18 hrs. No diminution in uric acid content was observed in 1 hr. extracts, 24 hr. extracts oxidised 6·1 mg., and 48 hr. extracts oxidised 7·2 mg. of uric acid. The residue left after extraction still contains extractable uricase, as was shown by the following experiment. 1 g. of dry uricase was extracted during 48 hrs. at 35° with 100 ml. of 0·125 % Na_2CO_3, filtered, the extract stored in a stoppered flask with toluene and the residue extracted as before with a fresh portion of Na_2CO_3, the second residue being again extracted. 5 ml. of the first extract and 5 ml. of Na_2CO_3 solution, and 10 ml. of the second and third, were left at 35° during 18 hrs. with 10 ml. of 0·13 % uric acid, after which uric acid was determined. 6·3 mg. of uric acid had been oxidised by the first extract, 5·4 mg. by the second and 1·0 mg. by the third, corresponding with an activity of 1·26 mg. per ml. of uricase in the first case, 0·54 mg. in the second and 0·1 mg. in the third.

2. *Comparison of activity of products and extracts.*

Hashed kidney tissue was shaken with 10 volumes of water, the suspension was filtered through cheese-cloth, and a part of the filtrate was treated according to Ro. The remainder was centrifuged, the centrifugate was filtered and the filtrate treated with acetone, *etc.*, by Ro's method; the residue was shaken successively twice with alcohol and twice with ether and then air-dried at 30°. The various products thus obtained were extracted with 0·125 % Na_2CO_3, and the activity of the extracts and the suspensions in 0·125 % Na_2CO_3 was determined.

The products examined were: Exp. 1, 1 % suspension of residue (I) obtained after centrifuging the suspension (III) of kidney tissue; Exp. 2, the

Table I. *Comparison of activity of products. All flasks contain 10 ml. of 0·104 % sodium urate and 10 ml. of uricolytic suspension, extract or 0·125 % Na_2CO_3.*

		mg. uric acid			
		After 18 hrs. at 35°		After 42 hrs. at 35°	
No. of Exp.	Product examined	Found	Oxidised	Found	Oxidised
Control	0·125 % Na_2CO_3.	10·4	—	10·4	—
1	1 % suspension of untreated uricase (I).	0·4	10·0	0	10·4
2	Filtered centrifugate (II) of tissue suspension (III).	10·2	0·2	8·2	2·2
3	1 % suspension of dry product (IV) obtained from (II).	10·0	0·4	9·2	1·2
4	0·17 % suspension of dry product (V) obtained from (I).	1·4	9·0	0	10·4
5	0·17 % suspension of dry product (VI) obtained from (III).	1·2	9·2	0	10·4
6	1 % extract of (IV).	10·4	0	10·4	0
7	„ „ „ (V).	0·6	9·8	0	10·4
8	„ „ „ (VI).	0·6	9·8	0	10·4
9	6 % „ „ (I).	10·0	0·4	9·6	0·8

filtered centrifugate (II); Exp. 3, a 1 % suspension of the dry product (IV) obtained by treating (II) according to Ro; Exp. 4, a 0·17 % suspension of the product (V) obtained by extracting 20 g. of (I) successively with two 200 ml. portions of 96 % alcohol, and with two 100 ml. portions of ether and drying at 35°; Exp. 5, a 0·17 % suspension of the dry product (VI) obtained by Ro's procedure; Exp. 6, 7 and 8, 1 % 24 hr. extracts of (IV), (V) and (VI); Exp. 9, a 6 % extract of (I). 1 % suspensions of (I) were compared with 0·17 % suspensions of (V) and (VI) in view of the fact that the dry content of (I) was about 15 %, as compared with 95 % in (V) and (VI).

It follows from the results obtained (Table I) that active products are obtained exclusively from material containing (I); *i.e.* uricolytic activity is associated exclusively with the insoluble parts of kidney tissue. The slight activity shown by the centrifugate and by extracts of (I) is ascribable to very fine, filter-passing particles of (I). A comparison of Exps. 1, 4 and 5 shows that the activity of (I) is very little affected by treatment with acetone, alcohol, ether and by drying. A comparison of Exps. 1, 7 and 8 shows that only a small part of the original activity of (I) is exhibited by the extracts, since the content of anhydrous material taken for extraction was 6 times greater in Exps. 7 and 8 than that present in Exp. 1. Exps. 4 and 5, 7 and 8 indicate that the uricolytic properties of Ro's product are practically identical with those obtained by treating (I) with alcohol and ether. Finally, Exp. 9 shows that uricolytic extracts cannot be obtained from (I) under conditions comparable with those of extraction of (V) and (VI).

3. *Simplified method of preparation of soluble uricase.*

The experiments described in the preceding section indicate that Ro's technique is unnecessarily complicated and results probably in the inclusion of a certain proportion of inactive matter in his dry products. The steps which appear to be superfluous are: 1. allowing the kidney-tissue suspension to stand with thymol; 2. treating the suspension with acetone. The following procedure was adopted in place of Ro's. Uricase is prepared as described in Part IV of this series, shaken during 2 mins. with 10 vols. of 96 % alcohol and rapidly filtered by suction; the process is repeated once more with alcohol and twice with the same volume of ether, after which the product is allowed to dry at room temperature, or at 37°. It is important that residual ether be allowed to evaporate spontaneously, without expressing on filter-paper, as the product obtained in the latter case is lumpy, black and yields relatively inactive extracts. The final product (this will in future be designated "dry uricase") is a white, impalpable powder, containing 5 % of moisture, and can be stored indefinitely. The yields of dry uricase average about 10 g. per kg. of kidney, as compared with 100 g. obtained by Ro. It is difficult to understand how such high yields could have been obtained, as the average dry content of ox-kidneys does not exceed 15–20 %, and by far the greater part of the raw material remains unsuspended. Moreover, of the solid matter of the suspension filterable through muslin, about 10 % is represented by lipoids, removed during the alcohol-ether extraction, and a further 10 % is present as water-soluble extractives. It must therefore be considered that, whilst somewhat higher yields than those obtained by the present author might be obtained by finer comminution of the original tissues, yet in no case could yields much higher than 1·5 % of dry uricase be expected without considerable contamination by inert matter. Solutions of uricase are obtained under the conditions described in section 1 of this paper.

4. *Significance of lipoid extractives.*

The combined alcoholic extracts from 100 g. of uricase were evaporated to dryness under reduced pressure at 38°, when about 3 g. of a semi-solid, oily, yellow residue, completely soluble in chloroform, were obtained. The combined ethereal extracts, similarly treated, yielded about 2 g. of a dry, white residue, M.P. 52–54°, also soluble in chloroform; 1 g. of each residue was dissolved in 100 ml. of ether, and 1 g. in chloroform. Three 0·2 g. portions of dry uricase were introduced into 100 ml. Erlenmeyer flasks, 5 ml. of the respective ethereal solutions or ether alone were added, and the ether was removed under reduced pressure at $\gtrless$ 37°. 20 ml. of 0·125 % Na_2CO_3 and 1 ml. of toluene were then added to each flask, their contents were filtered after 48 hrs., and the uricolytic activity of the extracts was determined as usual, taking 10 ml. of Na urate and 2 ml. of filtrate.

Table II.

No. of Exp.	Solution added to dry uricase	mg. uric acid: After 18 hrs. at 37° Found	After 18 hrs. at 37° Oxidised	After 66 hrs. at 37° Found	After 66 hrs. at 37° Oxidised	
1	5 ml. ether	3·4	7·4	0	10·8	
2	5 ml. ethereal solution of alcoholic extractives	10·8	·0	10·8	0	
3	5 ml. ethereal solution of ethereal extractives	10·8	0	10·8	0	
4	—	10·8	—	10·8	—	Control : 10 ml. sodium urate, 2 ml. 0·125 % Na_2CO_3.

The results obtained (Table II) indicate that the extractives removed by alcohol and by ether prevent extraction of uricase by aqueous Na_2CO_3, *i.e.* uricase is fixed on the tissues by the agency of lipoid substances (Exps. 2 and 3); Exp. 1 shows that the results obtained are not ascribable to inactivation of uricase by ether.

This conclusion as to the rôle of lipoids in the systems in question was further established by the following experiments. Three 0·5 g. portions of dry uricase were placed in three 100 ml. flasks; to one, 5 ml. of a 1 : 1 mixture of the chloroform solutions of the alcoholic and ethereal residues were added, to another

Table III.

No. of Exp.	Solution added to dry uricase	mg. uric acid after 24 hrs. at 37° Found	Oxidised	Remarks
1	5 ml. $CHCl_3$	0·2	9·0	
2	,,	0·2	9·0	Lipoids added to filtrate
3	5 ml. $CHCl_3$ solution of combined EtOH–Et_2O extractives	8·2	1·0	
4	5 ml. $CHCl_3$ solution cont. 5 mg. cholesterol	2·6	6·6	
5	—	9·2	—	Control : 10 ml. sodium urate, 10 ml. 0·125 % Na_2CO_3

5 ml. of chloroform containing 5 mg. of cholesterol, and to the third 5 ml. of chloroform. As before the solvent was evaporated at 37°, and the dry residues were extracted by 25 ml. of 0·125 % Na_2CO_3 during 48 hrs. at 37°. 10 ml. of each extract and 1 ml. of chloroform were then added to 10 ml. of sodium urate, and uric acid was determined after 24 hrs. at 37°; in addition, to a second 10 ml. portion of the extract of uricase to which lipoids had not been added, 1 ml. of the combined chloroform solution was added instead of pure chloroform.

The above experiments (Table III) confirm those of Table II; the slight activity shown by the extract in Exp. 3 as compared with the analogous experiment of Table II is probably ascribable to the greater concentration of uricase (10 times greater). Exp. 4 shows that the action of tissue lipoids is not necessarily specific, as the activity of the extract is reduced by 27 % by 5 mg. of cholesterol (50 mg. of lipoids were present in Exp. 3). Finally Exp. 2 proves that the lipoids do not inactivate extracted uricase, *i.e.* that their action consists in preventing extraction and not in inactivating the enzyme.

5. *Properties of soluble uricase.*

The extracts of soluble uricase are clear or slightly opalescent, straw-coloured liquids, possessing an odour similar to that of aliphatic amines and foaming on shaking. On keeping, they become turbid but do not lose their activity. Quite definite protein reactions are obtained (Millon, Adamkiewicz, biuret), and the N content amounts to 0·08–0·13 %. The p_H of the extracts is invariably less than that of the original Na_2CO_3 solution (p_H 9·12).

A preliminary survey of the properties of soluble uricase was made, preparatory to more systematic work. The first problem was that of further purification by means of filtration. A suspension of 1 g. of dry uricase in 100 ml. of aqueous Na_2CO_3 was allowed to stand during 24 hrs. at 35°, and 10 ml. of the well mixed suspension (I) were measured into a 100 ml. flask. The remainder of (I) was then filtered through filter-paper, 10 ml. of the filtrate were taken for examination, and the remainder was filtered through a Berkefeld filter; finally, a portion of this filtrate (III) was passed through a Bechhold ultra-filter (filtrate IV). 10 ml. of sodium urate solution and 1 ml. of toluene were added to 10 ml. of the various preparations examined, and uric acid was determined after 18 hrs. at 35°.

Table IV.

No. of Exp.	Nature of preparation	mg. uric acid after 18 hrs. at 35°	
		Found	Oxidised
1	Control	14·4	—
2	Suspension	0	14·4
3	Filtrate (II)	6·6	7·8
4	Berkefeld filtrate (III)	7·0	7·4
5	ditto, 24 hrs. later	7·0	7·4
6	ditto, refiltered	9·0	5·4
7	ditto, 4 days later	9·2	5·2
8	Bechhold filtrate (IV)	9·5	4·9

Filtrates (III) and (IV) were water-clear but (III) became turbid on standing during 24 hrs. at room temperature, without, however, losing any of its activity (Table IV, Exp. 5). Part of the activity is in the precipitate, as is shown by Exp. 6, in which the Berkefeld filtrate of the turbid extract was examined. The clear, second filtrate again became turbid on keeping a further 4 days, with slight inactivation (Exp. 7). The Bechhold ultra-filtrate, obtained using a membrane impermeable to ovalbumin, exhibited moderate activity (Exp. 8). Protein reactions were given by all filtrates, in decreasing order of intensity.

6. *Action of ammonium chloride and of methylamine hydrochloride on uric acid.*

In view of Schittenhelm and Chrometzka's [1927] contention that ammonia acts uricolytically, and of the ammoniacal odour of the extracts, it was thought desirable to investigate the possibility that the activity of the latter might be due to ammonia or methylamine. With this object, a number of systems were set up, consisting of 10 ml. of 0·1 % sodium urate, 10 ml. of 0·125 % Na_2CO_3, and 10 ml. of 0·08 % NH_4Cl, or of 0·02 %, 0·04 % or 1 % $CH_3.NH_2.HCl$. The uric acid content, determined after 24 hrs. and 48 hrs. at 37°, remained constant, pointing to the non-identity of uricase with these substances.

Discussion.

From the biological point of view the most interesting phenomenon of those described above is that of the action of lipoids in preventing extraction of uricase. Whilst it would be premature to enter into a detailed consideration of the mode of action of lipoids in this connection, yet it appears from a number of investigations now in progress in this laboratory that this binding action of lipoids is of considerably wider incidence than in the immobilisation of enzymes on tissues, evidence of its presence having been found in widely different biological processes. For the purposes of this paper, however, the conclusion may be drawn that biologically uricase acts as an insoluble enzyme.

The preliminary survey of the properties of soluble uricase confirms Ro's finding that uricolytic activity is indissociable from traces of alkali-soluble protein. This protein is of relatively small molecular weight, as it passes freely through an unglazed porcelain filter and partly through a membrane impervious to ovalbumin. Further work on soluble uricase will be described in the succeeding parts of this series.

Summary.

1. Ro's method [1931] for the preparation of soluble uricase has been investigated, and his results have been substantially confirmed.

2. The non-extractability of uricase from fresh tissues is attributable to the presence of lipoids; after their removal by the action successively of alcohol and ether, uricase may be extracted by dilute aqueous Na_2CO_3.

3. A consequence of the above finding is that uricase acts *in vivo* as a contact catalyst.

4. Dissolved uricase passes freely through Berkefeld filters, and partly through Bechhold filters, the membrane of which is impermeable to ovalbumin.

5. Uricolytic activity varies roughly in proportion to the intensity of the protein reactions of the solution.

6. Ammonium chloride and methylamine hydrochloride have no uricolytic action; soluble uricase is therefore not identical with these substances.

The author wishes to express his gratitude to Prof. St J. Przyłęcki for his helpful suggestions and criticism during the conduct of the above research.

REFERENCES.

Ro (1931). *J. Biochem.* **14**, 361.
Schittenhelm and Chrometzka (1927). *Z. physiol. Chem.* **162**, 203.
Truszkowski (1930). *Biochem. J.* **24**, 1341, 1349, 1359.
Wiechowski and Wiener (1907). *Beitr. chem. Physiol. Path.* **9**, 247.

XII. THE CONFIGURATIVE RELATIONSHIP OF THYROXINE AND TYROSINE.

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Since the constitution of thyroxine has been known it has been generally assumed that the compound is formed in the organism by the oxidative coupling of two molecules of 3 : 5-diiodotyrosine. The likelihood of such a reaction was indeed already referred to by Harington and Barger [1927] in the consideration of the then unknown orientation of the iodine atoms in the thyroxine molecule, and the hypothesis was considerably strengthened by the demonstration by Harington and Randall [1929] of the actual occurrence of 3 : 5-diiodotyrosine side by side with thyroxine in the thyroid gland.

Search which has been made in this and other laboratories for the possible existence of an enzymic mechanism capable of converting 3 : 5-diiodotyrosine into thyroxine has hitherto proved fruitless, so that direct biochemical evidence of the relationship of the two compounds is still lacking; nor is the hypothesis as yet susceptible of proof by biological means. There remains however a line of chemical investigation which is described in the present paper and of which the results appear to be conclusive.

It is evident that if thyroxine is in fact synthesised from 3 : 5-diiodotyrosine under biological conditions it must be configuratively related to the latter amino-acid and therefore also to tyrosine from which diiodotyrosine is undoubtedly derived. A chemical synthesis of thyroxine from natural *l*-tyrosine conducted by a series of reactions involving no racemisation and no possibility of a Walden inversion at any stage should lead therefore to the naturally occurring modification of thyroxine.

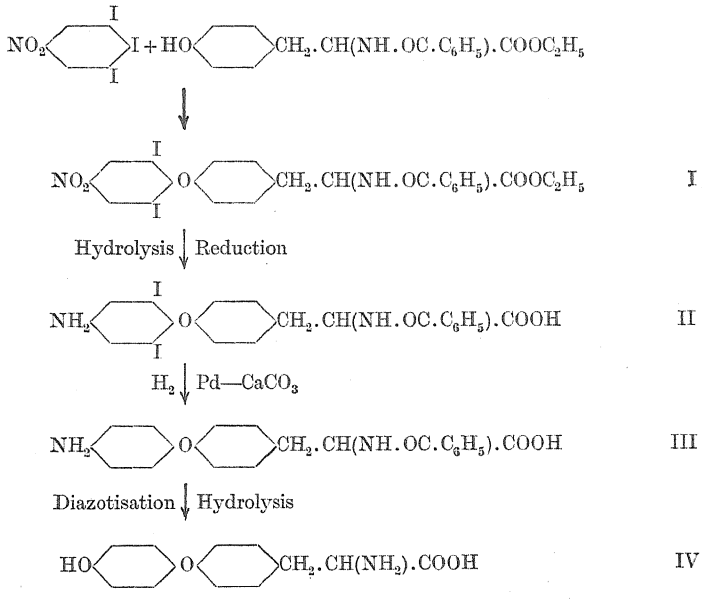
Unfortunately a direct synthesis of thyroxine itself from tyrosine or 3 : 5-diiodotyrosine is not feasible; on the one hand the halogenation of the thyronine nucleus in the 3 : 5-positions cannot be accomplished and on the other it is impossible to carry out the phenyl ether condensation with a phenolic substance such as 3 : 5-diiodotyrosine which is substituted in the *ortho*-positions to the hydroxyl group.

The problem has therefore had to be solved in an indirect manner. By a series of reactions to be described below it has been possible, starting from natural *l*-tyrosine, to synthesise an optically active thyronine having $[\alpha]_{5461}$ $+13{\cdot}3°$. Now we had in our hands some *l*-thyroxine which had been prepared by the resolution of *dl*-thyroxine according to Harington [1928] and which had been shown by the work of Harington and Salter [1930] to be identical with the naturally occurring compound; catalytic deiodination of a sample of this *l*-thyroxine yielded a preparation of thyronine having $[\alpha]_{5461}$ $+12{\cdot}2°$.

There can thus be no longer any doubt of the stereochemical relationship of natural *l*-thyroxine with natural *l*-tyrosine, and on chemical grounds at least we may feel secure in advancing the hypothesis that in nature thyroxine is derived from tyrosine.

Synthesis of d-*thyronine.*

The actual synthesis of *d*-thyronine from *l*-tyrosine was carried out as follows. The ethyl ester of *N*-benzoyl-*l*-tyrosine was condensed with 3 : 4 : 5-triiodonitrobenzene by boiling in methylethylketone solution with potassium carbonate to give ethyl α-benzamido-β-[4-(3′ : 5′-diiodo-4′-nitrophenoxy)phenyl]-propionate (I). (The object of employing 3 : 4 : 5-triiodonitrobenzene instead of the more



obvious *p*-iodonitrobenzene is simply to improve the yield at the stage of the condensation; the subsequent removal of the 3′ : 5′-iodine atoms offers no difficulties.) The ester (I) was hydrolysed to the free acid, which in turn was reduced with ferrous sulphate and baryta to the corresponding amino-compound (II); the latter was deiodinated by catalytic hydrogenation in presence of palladised calcium carbonate, and the resulting iodine-free amino-acid (III) was diazotised in dilute hydrochloric acid solution. The *N*-benzoylthyronine, which resulted on boiling the solution of the diazonium salt, was not isolated but was converted directly into *d*-thyronine (IV) by further boiling after the concentration of hydrochloric acid had been increased to 5*N*.

As already mentioned the thyronine obtained in this way had $[\alpha]_{5461}+13{\cdot}3°$, whilst a sample of thyronine obtained by the catalytic deiodination of *l*-thyroxine ($[\alpha]_{5461}-3{\cdot}5°$) had $[\alpha]_{5461}+12{\cdot}2°$; a third sample obtained by deiodination of *l*-3 : 5-diiodothyronine (itself related to *l*-thyroxine) had $[\alpha]_{5461}+13{\cdot}9°$.

Experimental.

N-benzoyl-l-tyrosine ethyl ester. This compound was most conveniently prepared as follows. *l*-Tyrosine ester hydrochloride (24·5 g.) was dissolved in the minimum amount of water; chloroform (200 ml.) was then added and the

mixture cooled to −10°. The free ester was liberated by addition of 5*N* sodium hydroxide (20 ml.) with vigorous shaking. Benzoyl chloride (14 g.) was then added in portions with shaking after each addition. The mixture was treated with 2*N* sodium carbonate (50 ml.) followed by a further equal amount of benzoyl chloride in portions. When evolution of CO_2 no longer occurred on shaking, the chloroform layer was separated, washed with water, dried over calcium chloride and evaporated; recrystallisation of the residue from a mixture of chloroform and light petroleum afforded 85 % of the theoretical amount of a product having M.P. 119°.

Ethyl α-benzamido-β-[4-(3′ : 5′-diiodo-4′-nitrophenoxy)phenyl]propionate (I). *N*-benzoyl-*l*-tyrosine ethyl ester (20 g.: 15 % excess) and 3 : 4 : 5-triiodonitrobenzene (28 g.) were dissolved in methylethylketone (200 ml.); freshly dried and finely powdered potassium carbonate (8·7 g.) was added and the mixture boiled under reflux for 18 hours. The solution was filtered and the precipitate extracted with hot methylethylketone; the combined filtrates were evaporated on the water-bath to a pasty residue which was triturated with cold water. The insoluble portion which was somewhat sticky was collected and recrystallised from 95 % alcohol (1800 ml.). The yield was 17·2 g. (45 % of the theoretical).

After recrystallisation from acetic acid the compound formed colourless prismatic needles, M.P. 189°. It had $[\alpha]_{5461}$ −1·8° (c=3·65 in acetic acid; 2 dm. tube). (Found: N, 4·0; I, 37·3 %. $C_{24}H_{20}O_6N_2I_2$ requires: N, 4·1; I, 37·0 %.)

α-Benzamido-β-[4-(3′ : 5′-diiodo-4′-nitrophenoxy)phenyl]propionic acid. The above ester (16 g.) was suspended in alcohol (55 ml.); 5·1*N* sodium hydroxide (5 ml.) was added and the mixture warmed on the water-bath with constant stirring; it became at first reddish in colour and more liquid; on further heating for a few minutes the colour disappeared and the consistency became thick and pasty. Water (200 ml.) was added and the mixture warmed until a clear solution was obtained; addition of hydrochloric acid precipitated the product, which was collected and crystallised from 70 % acetic acid. The yield was 14·7 g. and a further 0·75 g. was obtained by dilution of the mother-liquor with water.

For complete purification the acid was dissolved in methylethylketone and the filtered solution evaporated; the residue was then again crystallised from 70 % acetic acid. The compound formed colourless needles having M.P. 207°; it had $[\alpha]_{5461}$ −6·8° (c=2·72 in acetic acid; 2 dm. tube). (Found: N, 3·9; I, 38·4. $C_{22}H_{16}O_6N_2I_2$ requires: N, 4·3; I, 38·6 %.)

α-Benzamido-β-[4-(3′ : 5′-diiodo-4′-aminophenoxy)phenyl]propionic acid (II). The nitro-acid (11·7 g.) was dissolved in water (120 ml.) with the aid of sodium hydroxide sufficient just to make the solution alkaline to phenolphthalein. The solution was added to a suspension of ferrous hydroxide prepared by mixing a solution of hydrated ferrous sulphate (30 g.) in water (150 ml.) with one of hydrated barium hydroxide (38 g.) in water (100 ml.); the mixture was heated on the water-bath for 10 minutes. It was then brought to the boil, treated with a slight excess of sodium sulphate and filtered hot; the precipitate was extracted with boiling water and the combined solutions (about 1 litre) acidified with acetic acid and allowed to cool. The first crop amounted to 7·2 g. and a further 2·2 g. was obtained by working up the mother-liquor; the total yield was therefore 84 % of the theoretical. For analysis the acid was recrystallised from 50 % alcohol (charcoal) from which it separated in colourless needles having M.P. 197°. It had $[\alpha]_{5461}$ −15·0° (c=2·47 in a mixture of equal parts of *N* hydrochloric acid and alcohol; 2 dm. tube). (Found: N, 4·4; I, 39·8 %. $C_{22}H_{18}O_4N_2I_2$ requires: N, 4·5; I, 40·4 %.)

α-Benzamido-β-[4-(4'-aminophenoxy)phenyl]propionic acid (III). The preceding compound (9·75 g.) was dissolved in a mixture of *N* sodium hydroxide (280 ml.) and alcohol (200 ml.) and after addition of palladised calcium carbonate (15 g. [*cf.* Harington, 1926]) the solution was shaken in an atmosphere of hydrogen. Uptake of hydrogen ceased at 778 ml. (corrected; about 10 % in excess of the theoretical); the catalyst was filtered off and washed with 50 % alcohol. Alcohol was removed from the combined filtrates by distillation under diminished pressure and the aqueous residue acidified with acetic acid. The product was collected and dissolved in boiling water (200 ml.) with the aid of sodium acetate; the filtered solution was treated at the boiling-point with hydrochloric acid to give maximum precipitation. The yield was 5·1 g. (93 % of the theoretical).

For analysis the acid was recrystallised from 50 % alcohol; it formed colourless needles having M.P. 182·5°. It had $[\alpha]_{5461}-40{\cdot}0°$ ($c=2{\cdot}62$ in a mixture of equal parts of *N* hydrochloric acid and alcohol; 2 dm. tube). (Found: N, 7·1 %; $C_{22}H_{20}O_4N_2$ requires: N, 7·4 %.)

d-*Thyronine* (IV). The compound III (1·6 g.) was dissolved in 0·3 *N* hydrochloric acid (42·5 ml.) with the aid of heat; on cooling, the solution remained clear. Diazotisation was effected by the gradual addition of powdered sodium nitrite (0·3 g.) the solution being stirred and maintained at 0° throughout and for 15 minutes after the last addition; the diazonium salt crystallised in part. The mixture was diluted with water (40 ml.), and stirring was continued at 0° for 15 minutes longer; the whole was then transferred to a flask and boiled under reflux for 1·5 hours. An equal volume of concentrated hydrochloric acid was added and boiling continued for 6 hours more to effect hydrolysis of the benzoyl group. After cooling, the solution was diluted with an equal volume of water and extracted with ether to remove benzoic acid. The aqueous layer was evaporated to dryness under diminished pressure, and the residue was taken up in water (about 20 ml.); treatment of the hot aqueous solution with sodium acetate caused precipitation of the amino-acid. The yield was about 0·5 g.

The product was purified by dissolution in dilute sulphuric acid and reprecipitation from the hot solution by addition of sodium acetate. It had M.P. 249° (decomp.) and the M.P. was not affected by admixture with *d*-thyronine obtained from *l*-thyroxine (see below). The amino-acid had $[\alpha]_{5461}+13{\cdot}3°$ ($c=0{\cdot}75$ in a mixture of equal parts of *N* hydrochloric acid and alcohol; 2 dm. tube). (Found: N, 5·0 %. $C_{15}H_{15}O_4N$ requires: N, 5·1 %.)

d-Thyronine from l-*thyroxine.* *l*-Thyroxine (0·075 g.; $[\alpha]_{5461}-3{\cdot}5°$) was dissolved in *N* potassium hydroxide (7·5 ml.) and shaken in an atmosphere of hydrogen after addition of palladised calcium carbonate (0·25 g.); at the conclusion of the uptake of hydrogen the solution was filtered, acidified with acetic acid and evaporated on the water-bath; the recrystallised thyronine amounted to 0·021 g. It had M.P. 247° (decomp.) and $[\alpha]_{5461}+12{\cdot}2°$ ($c=4{\cdot}12$ in a mixture of equal parts of *N* hydrochloric acid and alcohol; 0·5 dm. micro-tube). (Found: N, 5·3 %. $C_{15}H_{15}O_4N$ requires: N, 5·1 %.)

d-*Thyronine from* l-*3 : 5-diiodothyronine.* For further confirmation a preparation of thyronine was made from *l*-3 : 5-diiodothyronine which is itself known to be stereochemically related to *l*-thyroxine [*cf.* Harington, 1928]. *l*-3 : 5-Diiodothyronine (0·3 g.; $[\alpha]_{5461}-1{\cdot}0°$) was catalytically deiodinated in presence of palladised calcium carbonate (1 g.); the yield of thyronine was 0·128 g. and the product had M.P. 252° (decomp.) and $[\alpha]_{5461}+13{\cdot}9°$ ($c=0{\cdot}753$ in a mixture of equal parts of *N* hydrochloric acid and alcohol; 2 dm. tube). (Found: N, 5·1 %. $C_{15}H_{15}O_4N$ requires: N, 5·1 %.)

Note on preparation of l-*4-(4′-aminophenoxy)phenylalanine.*

α-Benzamido-β-[4-(4′-aminophenoxy)phenyl]propionic acid (III) (0·5 g.) was boiled under reflux for 4 hours with 20 % hydrochloric acid (10 ml.); the solution was cooled, diluted with water and extracted with ether to remove benzoic acid. The aqueous layer was evaporated to dryness under diminished pressure and the residue taken up in a little hot water. After partial neutralisation with sodium acetate the solution was boiled with charcoal and filtered and the filtrate carefully neutralised to litmus. The product which separated on cooling was collected and recrystallised from boiling water from which it separated in colourless glistening needles. It had M.P. 239° (decomp.) and $[\alpha]_{5461} - 5{\cdot}0°$ ($c = 1{\cdot}2$ in $0{\cdot}5N$ hydrochloric acid; 1 dm. tube). The compound was considerably more soluble than thyronine in water; it gave a very intense ninhydrin reaction without previous neutralisation with sodium acetate. (Found: N, 10·3 %. $C_{15}H_{16}O_3N_2$ requires: N, 10·3 %.)

REFERENCES.

Harington (1926). *Biochem. J.* **20**, 300.
—— (1928). *Biochem. J.* **22**, 1429.
—— and Barger (1927). *Biochem. J.* **21**, 169.
—— and Randall (1929). *Biochem. J.* **23**, 373.
—— and Salter (1930). *Biochem. J.* **24**, 456.

XIII. THE COMPONENT FATTY ACIDS AND GLYCERIDES OF THE MILK-FAT OF INDIAN CAMELS.

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THE component fatty acids and glycerides of the milk-fat of the camel have been studied in detail, in order to compare them with those of the milk-fats of the cow [Hilditch *et al.*, 1929; 1930; 1931], buffalo [Bhattacharya and Hilditch, 1931], sheep and goat [Dhingra, 1933].

In north-western parts of India milk from camels is utilised as an article of diet by some tribes, but the fat is rarely extracted from the milk and used as a substitute for "ghee" (camel milk-fat is not included in the term "ghee" as defined in the Bombay "Adulteration of Ghee" Act).

The milk-fat used in the present investigation was prepared by churning fresh milk from the animals, and after separating water and caseinogen from the butter, the fat was stored *in vacuo* in sealed amber glass bottles. The milk-fat was obtained in the month of March 1933, when the temperature range was 50°–80° F., from Gujranwala (Panjab) camels, which had been fed partly on green grass but mainly on the tree leaves of *Zizyphus jujuba*, *Acacia speciosa* and *Acacia Arabica*. The yield of the fat from different samples of the milk varied from 2·0 to 2·5 %.

The general characteristics of the milk-fat are given in Table I.

Table I.

Colour	Almost white (cream tinge)
Odour	Buttery, with a slight tallowy odour
Sap. equiv.	259·0
Iodine value	40·8
Acid value	0·2
Reichert-Meissl value	16·4
Polenske value	1·6
Kirschner value	14·3
Setting-point	35·3
Refractive index $n_D^{40°}$	1·4555

The Reichert-Meissl and Kirschner values of camel butter-fat are lower and the saponification equivalent is higher than corresponding characteristics of cow, buffalo, goat and sheep butter-fats, but the Polenske value is considerably lower than that of the goat and sheep butter-fats and more nearly resembles that of cow and buffalo milk-fats.

Component fatty acids of camel milk-fat.

The method followed for the quantitative determination of the component fatty acids was that developed by Hilditch and Jones [1929]. On account of the small amount of the fat available, the mixed fatty acids from 200 g. of fat only

were employed for this purpose. The details of fractionation data are given in Table II, and the final results for the fat are summarised in Table III.

Table II.

(*a*) *Acids volatile in steam.*

No.	g.	B.P. (° C.)	Pressure	Mean equivalent	Acids: Butyric	Caproic	Caprylic	Capric	Oleic	N – S
1	In aqueous solution (4580 ml.)			—	0·90	—	—	—	—	—
2	In recovered ether (1535 ml.)			—	0·05	—	—	—	—	—
3	4·99	35–80	Atmospheric	—	0·13	—	—	—	—	—
4	1·55	80–140	,,	—	0·65	—	—	—	—	—
5	1·02	141–142	,,	—	0·91	—	—	—	—	—
6	1·22	140–141	,,	95·7	0·83	0·39	—	—	—	—
7	0·88	142–160	,,	101·0	0·42	0·46	—	—	—	—
8	0·57	100–110	Reduced	107·7	0·14	0·43	—	—	—	—
9	0·68	112–122	,,	126·4	—	0·40	0·28	—	—	—
10	1·48	Residue	,, (Iodine value 13·0)	183·2	—	—	0·94	0·14	0·21	0·19
					4·03	1·68	1·22	0·14	0·21	0·19

(*b*) *Acids non-volatile in steam.* 190 g.

(Sap. equiv. 261·9; Iod. val. 43·4.)

Lead salt separation.

	g.	%	Corresponding esters: S.E.	I.V.
"Solid" acids S	71·6	48·9	276·1	6·2
"Liquid" acids L	74·8	51·1	274·4	73·9

(*c*) *Fractionation of methyl esters.*

(i) Esters of "Solid" acids S.

Primary fractions: No.	g.	B.P./2 mm.	sap. eq.	I.V.	Refractionations: No.	g.	B.P./2 mm.	sap. eq.	I.V.
					S 11	3·73	120–156	261·0	2·3
					S 12	6·13	155–157	265·2	2·8
S 1	31·96	130–162	270·3	4·0	S 13	4·47	155–157	267·9	3·1
					S 14	4·27	156–158	273·0	4·0
					S 15	5·68	160–168	276·1	5·5
S 2	4·81	160–164	283·4	8·2	S 16	2·40	Residue	283·9	8·7
S 3	4·45	165–167	290·2	10·7					
S 4	3·71	Residue	297·8	15·3					

(ii) Esters of "Liquid" acids L.

					L 11	2·17	95–133	214·0	26·8
					L 12	4·10	135–148	250·7	47·4
					L 13	4·70	148–155	267·5	59·7
L 1	38·94	95–180	270·1	70·2	L 14	5·12	152–159	270·3	64·4
					L 15	5·13	158–160	281·9	78·2
					L 16	3·24	158–161	283·3	83·9
					L 17	4·80	158–160	295·8	88·9
					L 18	3·20	Residue	295·1	90·1
L 2	3·85	178–181	296·3	93·5					
L 3	2·00	181–182	295·9	94·2					
L 4	3·28	Residue	308·4	84·6					

The presence of the ordinary $\Delta^{9:10}$-oleic and $\Delta^{9:10,12:13}$-linoleic acids in the unsaturated components of the fat was shown by the identification of a dihydroxy-

Table III. *Summarised experimental data for fatty acids.*

Acid	Volatile acids 3·9 %	Acids non-volatile in steam: Solid acids S 47·0 %	Acids non-volatile in steam: Liquid acids L 49·1 %	Component fatty acids including unsaponifiable % (wt.)	Component fatty acids excluding unsaponifiable % (wt.)	Component fatty acids excluding unsaponifiable % (mols.)
Butyric	2·10	—	—	2·10	2·1	5·9
Caproic	0·88	—	—	0·88	0·9	1·9
Caprylic	0·64	—	—	0·64	0·6	1·1
Capric	0·07	—	1·36	1·43	1·4	2·1
Lauric	—	—	4·56	4·56	4·6	5·7
Myristic	—	3·35	3·93	7·28	7·3	7·9
Palmitic	—	29·22	—	29·22	29·3	28·3
Stearic	—	11·08	—	11·08	11·1	9·7
Oleic	0·11	3·35	35·34	38·80	38·9	34·1
Linoleic	—	—	3·77	3·77	3·8	3·3
Unsaponifiable	0·10	—	0·14	0·24	—	—

stearic acid (M.P. 130°) and a tetrahydroxystearic acid (M.P. 172°) respectively from the products of oxidation of the dilute aqueous solution of the potassium salts of the acids from the penultimate fraction of the "liquid" esters. On hydrolysis of the residual fraction of the "solid" esters, stearic acid was identified.

For comparison, the molar percentages of the component fatty acids of one buffalo, two cow and one sheep milk-fats, along with those of the camel butter, are given in Table IV; while additional information may be gained from consideration of the combined amounts of the four groups of acids given at the foot of the Table.

Table IV. *Comparison of the molar distribution of the component acids of camel, cow, buffalo and sheep milk-fats.*

Fat	Camel butter	Cow butters [Hilditch and Sleightholme, 1931]: Autumn fed English I	Cow butters [Hilditch and Sleightholme, 1931]: Early summer pasture-fed English IV	Sheep butter [Dhingra, 1933]	Buffalo butter No. 2 [Bhattacharya and Hilditch, 1931]
Iodine value	40·8	41·3	41·6	32·1	33·5
Butyric	5·9	8·4	8·9	8·4	11·0
Caproic	1·9	3·5	2·7	5·4	2·8
Caprylic	1·1	2·7	2·0	5·8	1·5
Capric	2·1	2·9	3·0	10·1	2·3
Lauric	5·7	4·1	4·7	6·0	3·3
Myristic	7·9	7·2	10·9	11·8	10·4
Palmitic	28·3	27·1	24·3	20·4	28·7
Stearic	9·7	6·4	5·4	5·4	9·3
Arachidic	—	0·7	—	1·3	0·7
Oleic	34·1	33·9	34·6	22·2	27·8
Linoleic	3·3	3·1	3·5	3·2	2·2
Butyric-lauric	16·7	21·6	21·3	35·7	20·9
Myristic-palmitic	36·2	34·3	35·2	32·2	39·1
Stearic (-arachidic)	9·7	7·1	5·4	6·7	10·0
Oleic-linoleic	37·4	37·0	38·1	25·4	30·0

It is clear that camel milk-fat contains definitely less butyric acid than any of the other milk-fats in this Table. The caprylic and capric acid contents of camel milk-fat resemble those of the milk-fats of the cow and buffalo; sheep and goat

milk-fats, it may be recalled, stand apart in their relatively high proportions of these two acids and in corresponding reductions in their contents of oleic and palmitic acids. The differences between the fatty acids of the camel milk-fat and milk-fats of autumn-fed and early summer pasture-fed English cows is not so marked as between those of the former and the milk-fats of the buffalo, goat or sheep.

The low content of acids volatile in steam in camel milk-fat may partly be due to its xerophytic plant food, or as suggested by Dhingra [1933] in the case of goat and sheep butters, it may be mainly connected with the species of the animal. Possibly the amount of these acids decreases with the size and species of the animals in the decreasing order of sheep, goat, cow, buffalo and camel; but this conclusion requires further confirmation and for this purpose experiments are in progress.

Component glycerides of the camel milk-fat.

The glyceride structure of the fat was studied by separating quantitatively the fully-saturated glycerides present and by determining the component fatty acids in the latter, as described by Hilditch and Jones [1929]; from the combined data for the component fatty acids of the whole fat and of the fully-saturated glycerides, the components of the mixed saturated-unsaturated glycerides present were deduced.

The fat (250 g.) yielded after oxidation crude neutral products (69·9 g.), a portion of which (69·0 g.) was resolved by further purification into: (*a*) 56·2 g., acid value 0·3, (*b*) 4·5 g., acid value 16·1, and (*c*) acidic products, 8·3 g., acid value 90·3. From this, the amount of fully-saturated glycerides in the original fat was determined to be 24·2 % (wt.) or 25·6 % (mols.), corresponding to an "association ratio" of 0·98 mol. of saturated per mol. of unsaturated acids in the mixed saturated-unsaturated glycerides. The component acids of the fully-saturated glycerides are made up as follows:

Table V.

Acid	% (wt.) (Excluding unsaponifiable matter)	% (mol.) (Excluding unsaponifiable matter)
Butyric	3·4	8·9
Caproic	1·0	2·1
Caprylic	0·1	0·1
Capric	2·1	2·8
Lauric	2·2	2·6
Myristic	18·4	18·9
Palmitic	55·0	50·0
Stearic	17·8	14·6

As in cow, goat and sheep butter-fats, the proportion of fully-saturated components of camel milk-fat is a function of the relative amounts of saturated and unsaturated acids in the whole fat. The amount of the fully-saturated glycerides in the present case is somewhat less for a given ratio of saturated to unsaturated acids in the whole fat, than that in cow butters of somewhat similar iodine value. It will be noticed also that the ratio of saturated to unsaturated acids in the mixed saturated-unsaturated glycerides of camel milk-fat is somewhat higher than that of cow butters.

Distribution of the fatty acids in the camel butter glycerides.

The molar percentages of the different groups of fatty acids in the whole fat, and its fully-saturated and non-fully-saturated glyceride components are collected in Table VI. As in Table IV, the data for the milk-fats of two cows, buffalo and sheep are also added for comparison with the camel milk-fat.

Table VI. *Molar distribution of fatty acids in camel, cow, buffalo and sheep milk-fats.*

Section	Acids	Camel butter	Cow butters [Hilditch and Sleightholme, 1931] Autumn fed English I	Cow butters [Hilditch and Sleightholme, 1931] Early summer pasture English IV	[Bhattacharya and Hilditch, 1931] Buffalo ghee, No. 2	[Dhingra, 1933] Sheep butter
Whole fat	Butyric-lauric	16·7	21·6	21·3	20·9	35·7
	Myristic	7·9	7·2	10·9	10·4	11·8
	Palmitic	28·3	27·1	24·3	28·7	20·4
	Stearic (-arachidic)	9·7	7·1	5·4	10·0	6·7
	Oleic-linoleic	37·4	37·0	38·1	30·0	25·4
Fully-saturated	Molar % F.S.G.	25·6	29·1	27·2	34·0	36·8
	Butyric-lauric	16·5	28·6	30·5	25·9	48·3
	Myristic	18·9	13·2	15·1	9·2	15·9
	Palmitic	50·0	43·1	39·5	47·1	26·9
	Stearic (-arachidic)	14·6	15·1	14·9	17·8	8·9
Mixed saturated-unsaturated	Molar % F.S.G.	74·4	70·9	72·8	66·0	67·2
	Butyric-lauric	16·6	19·0	18·3	18·5	28·4
	Myristic	4·2	5·0	9·4	11·1	9·4
	Palmitic	20·8	20·9	18·7	19·2	16·5
	Stearic (-arachidic)	8·0	4·1	2·2	5·8	5·5
	Oleic-linoleic	50·4	51·0	51·4	45·4	40·2

Hilditch and Sleightholme [1931] observed that the component acids of fully-saturated glycerides of six pasture-fed cow butter-fats, which differed widely in iodine value, were almost constant; but the corresponding data for camel milk-fat differ widely from all these, except that the percentage of stearic acid reaches a figure of about 15 % in camel and also in most of the cow milk-fat fully-saturated glycerides. The main differences between the two classes of fully-saturated glycerides are thus the lower percentage of the butyric-lauric acid group and the correspondingly higher proportions of myristic and palmitic acids in the case of the camel butter-fat. The differences between the components of the fully-saturated glycerides of the milk-fat of camels and those of goat, sheep and buffalo butter-fats are much more marked.

The percentages of the components of the mixed saturated-unsaturated glycerides of camel milk-fat are on the whole similar to those of the six pasture-fed cow butters and, to some extent, to those of buffalo ghee; but they differ from those of goat and sheep milk-fats in showing a lower percentage of the butyric-lauric group of acids and a correspondingly higher proportion of unsaturated acids.

SUMMARY.

The fatty acids of the milk-fat of the camel differ from those of cow, buffalo, goat and sheep milk-fats in a lower content of fatty acids volatile in steam. Camel milk-fat can to a great extent be distinguished from the milk-fats of other animals by its lower Kirschner and Reichert-Meissl values. The amount of the lower fatty acids is in decreasing order in the milk-fats of the sheep, goat, cow, buffalo and camel.

The content of the fully-saturated glycerides in camel milk-fat is a function of mean unsaturation, like that of the milk-fats of other animals. The component fatty acids of the fully-saturated glycerides of camel milk-fat are in somewhat different proportions to those in the milk-fats hitherto studied. The composition of the fatty acids of the mixed saturated-unsaturated glycerides of camel milk-fat is little different from that of most cow and buffalo butters, but more so from that of goat and sheep milk-fats.

From the point of view of general properties and of the component glycerides present, there seems no reason why camel milk-fat should not be used as a substitute for cow, buffalo, *etc.*, milk-fats.

REFERENCES.

Bhattacharya and Hilditch (1931). *Analyst*, **56**, 161.
Dhingra (1933). *Biochem. J.* **27**, 851.
Hilditch and Jones (1929). *Analyst*, **54**, 75.
—— and Sleightholme (1930). *Biochem. J.* **24**, 1098.
—— —— (1931). *Biochem. J.* **25**, 507.

XIV. A MODIFIED SPECTROPHOTOMETRIC METHOD FOR THE ASSAY OF CAROTENE AND VITAMIN A IN BUTTER.

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In a previous investigation [Gillam *et al.*, 1933] a spectrophotometric method was developed for the evaluation of carotene, xanthophyll and vitamin A in butter. This involved the separation of xanthophyll from carotene by the well-known partition between methyl alcohol and light petroleum, but although necessary for an original research, it was felt that this method could now be considerably shortened for routine purposes. Experience shows that the carotene : xanthophyll ratio in butter averages 14 : 1 by weight, a fact which, as will be seen later, permits of the approximate estimation of the relatively unimportant xanthophyll without actual determination.

The spectrophotometric evaluation of carotene and xanthophyll depends on the fact that each of these substances exhibits a group of absorption bands in the visible region of the spectrum, the principal one occurring at 463 $m\mu$ for carotene and 455 $m\mu$ for xanthophyll (in chloroform solution). The amount of either carotenoid present in a solution containing only one of them is proportional to the intensity of its particular absorption band.

The following table shows the average light absorptive power (which is related to the yellow colour) of the carotene and xanthophyll fractions of some 70 samples of English butter, arranged in six sets but actually representing twenty small groups of cattle, of several pure breeds.

Group	No. of samples	*$E^{1\%\text{ dry butter}}_{1\text{ cm.}}$ due to carotene (463 $m\mu$)	$E^{1\%\text{ dry butter}}_{1\text{ cm.}}$ due to xanthophyll (455 $m\mu$)	% of total absorption due to carotene (460 $m\mu$)
A	11	0·00522	0·000348	93·8
B	9	0·00395	0·000285	93·3
C	10	0·00878	0·000468	94·9
D	8	0·00526	0·000333	94·1
E	24	0·00502	0·000371	93·1
F	8	0·01159	0·000391	96·7
	70		Average value	94·0

* $E^{1\%}_{1\text{ cm.}} = \log I_0/I$ for a 1 cm. layer of a solution equivalent to 1 % of dry butter, I_0 = the intensity of the incident light, and I = the intensity of the transmitted light.

Allowing for the slightly different intensities of absorption of carotene and xanthophyll ($E^{1\%}_{1\text{ cm.}}$ 463 $m\mu$ = 1900 for carotene, and $E^{1\%}_{1\text{ cm.}}$ 455 $m\mu$ = 1700 for xanthophyll (lutein), when chloroform is the solvent) this absorption ratio of 94 : 6 becomes 14 : 1 for the ratio of carotene to xanthophyll by weight in butter.

These results, and the individual analyses from which they were summarised, show that:

(*a*) On the average, 94 % of the light absorption of butter-unsaponifiable matter in chloroform, at 455–460 $m\mu$, is due to carotene, the remainder being due to xanthophyll.

(*b*) The values for individual samples approximate to this mean value, and as xanthophyll is only a minor constituent of the butter pigment, small departures from constancy do not affect the resulting carotene percentage seriously, if the xanthophyll is estimated at approximately 6 % of the total yellow colour, instead of being actually determined.

The estimation of the carotene value at approximately 94 % of the total yellow colour of the butter, makes it possible to modify considerably the method of evaluation of carotene and vitamin A previously developed, with advantages which more than offset any error likely to occur through departures from constancy in the carotene : xanthophyll ratio.

(*a*) The elimination of the xanthophyll determination removes the need for the partition between light petroleum and methyl alcohol, with consequent saving of much time and material.

(*b*) The spectroscopic work is reduced to half because the unsaponifiable matter is examined as a whole instead of in two separate fractions.

(*c*) The accuracy of the method is increased by the much reduced handling of material well-known to be labile, as well as by the evaluation of the vitamin A in one fraction, instead of adding two results (and therefore two errors) together.

(*d*) The weight of butter required for the determination can be reduced, making a further economy in time and material.

Baumann and Steenbock [1933] have recently reported the results of an examination of fourteen American butters consisting of bulked samples representing the butter obtained in their particular district in each month of the year. They used a spectrophotometric method for both the carotene and vitamin A determinations but did not consider it necessary to correct their carotene values for xanthophyll. They pointed out, however, that in the case of the unsaponifiable matter of one particular August butter, approximately 95 % of the pigment was not extractable from light petroleum by methyl alcohol, and moreover, its absorption spectrum was characteristic of carotene. Thus the value they found for the carotene : xanthophyll ratio in this particular butter is very close to the value here shown to be the average of a large number of butters.

The modified procedure.

Preparation of the unsaponifiable matter. A weighed portion of the butter (100 g. of winter butter, or 25–50 g. of summer butter or half quantities if the antimony trichloride test is not being used) of known moisture content, is saponified for half an hour with a slight excess of colourless 20 % alcoholic potash, and the soaps are poured into about three volumes of water[1]. The solution is extracted with ether until no more colour is removed, the extracts being bulked and washed carefully with several portions of water. The whole of the washed ether extract is then freed from solvent by evaporation in nitrogen. If the spectroscopic examination is not to be carried out at once this evaporation should be completed in a brown glass ampoule, the residue being finally sealed off in

[1] The colourless alcoholic potash recommended is obtained by using alcohol which has been previously boiled over, and distilled from, either metallic sodium or caustic soda.

nitrogen. Otherwise, the residue is dissolved in chloroform, made up to 10–20 ml. in a graduated flask and examined immediately.

Determination of the carotene. The solution now represents a known weight of butter per 100 ml. The intensity of absorption at 455–460 $m\mu$ is measured either photographically, using an apparatus such as the Hilger E3 quartz spectrograph with a suitable photometer, or visually, using a spectrophotometer of the type of the Hilger-Nutting instrument[1].

We have found the visual method to be the better of these two, as there is less danger of destruction of the carotenoids by the ultra-violet rays of the light source (where an arc or spark is used with quartz apparatus) in the photographic method.

The value so obtained is expressed as $E^{1\%\ \text{butter}}_{1\ \text{cm.}}$ at 455–460 $m\mu$ and the absorption due to carotene is taken as 94 % of this value. To calculate the percentage of carotene in the butter, use is made of the fact that $E^{1\%}_{1\ \text{cm.}}$ 463 $m\mu$ ($CHCl_3$) = 1900 for pure carotene [Gillam *et al.*, 1932]. The result is conveniently expressed as mg. carotene per 100 g. dry butter.

Determination of the vitamin A.

(*a*) *By means of the ultra-violet absorption band.* It is now generally accepted that the absorption band at 328 $m\mu$ shown by certain fish-liver oils [Morton and Heilbron, 1928] is a definite property of vitamin A. This is also exhibited by butter [Morton and Heilbron, 1930] and provides a means of evaluation of the vitamin A content. In the case of butter-unsaponifiable matter the absorption at 328 $m\mu$ is a summation of that due to vitamin A together with the smaller contributions due to carotene and xanthophyll. We have found that the absorption due to carotene *plus* xanthophyll, at 328 $m\mu$, is given by dividing the observed value of $E^{1\%}_{1\ \text{cm.}}$ for these substances at 455–460 $m\mu$ by the factor 6·5. This correction is usually of the order 10–25 % of the gross absorption at 328 $m\mu$. From the value of $E^{1\%\ \text{butter}}_{1\ \text{cm.}}$ at 328 $m\mu$, due to vitamin A, it is possible to determine the vitamin A content in mg. per 100 g. dry butter by making use of the value $E^{1\%}_{1\ \text{cm.}} = 1600$ given by Carr and Jewell [1933] for their richest concentrate. This value may need correction if vitamin A is concentrated further (*cf.* Karrer and Morf [1933], who have prepared a concentrate of $E^{1\%}_{1\ \text{cm.}}$ (328 $m\mu$) = 1700).

(*b*) *By the spectroscopic blue test with antimony trichloride.* If the solution of butter-unsaponifiable matter is treated with antimony chloride in chloroform the blue colour formed is largely due to vitamin A, but some small part is also due to carotene and, to a lesser extent, to xanthophyll. If, however, the intensity of absorption of the blue solution is determined at 585 $m\mu$ with a visual spectrophotometer, according to the technique already described for liver oils [Heilbron *et al.*, 1931] and applied to butters [Gillam *et al.*, 1933], the value so obtained can be regarded as being due to vitamin A and carotene only, the xanthophyll value here being negligible. As therefore the absolute carotene value is known from the direct absorption and as the blue value of pure carotene is also known ($E^{1\%}_{1\ \text{cm.}}$ 585 $m\mu$ = 420), it is possible to calculate the contribution due to carotene. The difference gives the blue value due to vitamin A in terms of $E^{1\%\ \text{butter}}_{1\ \text{cm.}}$ at 585 $m\mu$, and as the purest distillates have a value $E^{1\%}_{1\ \text{cm.}}$ 585 $m\mu$ = 2600 [Heilbron *et al.*, 1932] in chloroformic antimony trichloride, the vitamin A content can be

[1] Although pure carotene has its principal absorption band at 463 $m\mu$ for chloroform solution, in the presence of butter-unsaponifiable matter this lies nearer to 455 $m\mu$. The intensity of absorption however is not perceptibly altered by taking the measurement anywhere between 455 and 460 $m\mu$.

calculated in mg. per 100 g. butter. The results obtained in this way are always low, apparently owing to the presence in the original butter of substances which act as inhibitors of the blue colour. This is in agreement with the findings of Booth *et al.* [1933] who have shown that the blue values of butter (in Lovibond units) exhibit considerable inhibition and that the inhibition shows a seasonal variation.

In view of these facts the spectroscopic modification of the antimony trichloride blue test is not recommended for the general determination of the vitamin A content of butter, but it is included here as a secondary test if for any reason, such as the presence of unsaponifiable artificial colouring matter, the ultra-violet absorption spectrum method is interfered with.

An analysis of the carotene, xanthophyll and vitamin A values of some 70 English butters consisting largely of winter samples yields the following data (obtained by the full method):

	mg. per 100 g. dry butter			Estimated growth-promoting effect* in International units per g.
	Carotene (1900 basis)	Xanthophyll (1700 basis)	Vitamin A (1600 basis)	
Mean values (70 samples)	0·327	0·024	0·473	11·1
Maximum	0·88	0·075	0·922	24·2
Minimum	0·025	0·003	0·158	2·9
Mean value cows at grass (12 samples)	0·60	0·029	0·72	18·0
Mean value stall-fed cattle (58 samples)	0·272	0·0227	0·420	9·7

* Calculated from the numerical values on the assumption that vitamin A is 1·66 times as potent as carotene [Carr and Jewell, 1933]. This value may need revision when really representative values are available.

In connection with the application of this method the following points should be noted.

(*a*) All values recorded were obtained on unsalted butters and are expressed on dry butter-fat. To obtain comparable results on salted butter the water and salt should be estimated and the carotenoid values calculated on the butter-fat.

(*b*) The majority of these butters were made from the milk of pure-bred Shorthorn cows, with a sufficient number of samples from pure-bred Ayrshire and South Devon herds to show that the carotene : xanthophyll ratio applies approximately to these also. A few unclassified samples have also been tested with good results.

(*c*) When butters contain added colouring matter, any method for carotenoid determination depending on yellow colour will give faulty results, hence the treatment will need modification according to the nature of the colouring matter. If this should interfere with the absorption spectrum determination of vitamin A, the antimony chloride test can be called into service.

Summary.

1. Values for the carotene, xanthophyll and vitamin A contents of seventy English butters have been obtained spectrophotometrically. Mean values are recorded.

2. It is shown that, on the average, the ratio of carotene to xanthophyll in butter is 14 : 1 by weight.

3. It is further pointed out that if 94 % of the light absorption of butter-unsaponifiable matter at 455–460$m\mu$ is taken as being due to carotene, the results on individual samples are sufficiently close to those actually determined after removal of the xanthophyll to justify the approximation by the economy effected in time and material.

4. The spectrophotometric method previously developed for the determination of carotene and vitamin A in butter has been improved for routine purposes. Full details of the modified method are given.

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REFERENCES.

Baumann and Steenbock (1933). *J. Biol. Chem.* **101**, 547.
Booth, Kon, Dann and Moore (1933). *Biochem. J.* **27**, 1189.
Carr and Jewell (1933), *Nature*, **131**, 92.
Gillam, Heilbron, Morton and Drummond (1932). *Biochem. J.* **26**, 1174.
—— —— —— Bishop and Drummond (1933). *Biochem. J.* **27**, 878.
Heilbron, Gillam and Morton (1931). *Biochem. J.* **25**, 1352.
—— Heslop, Morton, Webster, Rea and Drummond (1932). *Biochem. J.* **26**, 1178.
Karrer and Morf (1933). *Helv. Chim. Acta*, **16**, 625.
Morton and Heilbron (1928). *Biochem. J.* **22**, 987.
—— —— (1930). *Biochem. J.* **24**, 870.

XV. THE NUCLEOTIDE COMPONENT OF MAMMALIAN CARDIAC MUSCLE.

I. THE COMPOSITION AND CHEMICAL PROPERTIES OF THE NUCLEOTIDE.

By FLORENCE BEATTIE, THOMAS HUGH MILROY and ROBERT WILLIAM MAGILL STRAIN.

II. THE BIOLOGICAL AND CO-FERMENT PROPERTIES OF THE CARDIAC DINUCLEOTIDE.

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I. THE COMPOSITION AND CHEMICAL PROPERTIES OF THE NUCLEOTIDE.

The presence of an adenosinetriphosphoric acid in mammalian skeletal muscle was discovered independently by Fiske and Subbarow [1929] and Lohmann [1929]. Barrenscheen and Filz [1932] have recently suggested that the labile phosphoryl linkages in this nucleotide are at least in part with the NH_2 of the adenine, their opinion being based mainly upon the difficulties associated with the deamination without dephosphorylation (of the loosely bound phosphorus), whether this is brought about by chemical or by enzymic processes. From an examination of their acid hydrolysis experiments it is evident that the nucleotide which they had prepared from skeletal muscle behaved differently from pure adenosinetriphosphoric acid for, even after 1–3 hours' treatment with *N* HCl at 100°, only 80–85 % of the loosely bound phosphoric acid was set free, while the same compound prepared by others readily gives off completely two of the three phosphoric acid molecules within ten minutes under the above conditions. From a study of the acid hydrolysis curves of nucleotides certain important differences in the nature of the linkages in purine nucleotides of different origin may be readily observed. Even in the cases of the adenosinemonophosphoric acids of yeast and muscle, it is evident that there is a characteristic difference in the firmness of the single phosphoryl linkage with an alcohol group of the pentose. Thus the phosphoryl linkage of the yeast derivative can be readily broken by acid hydrolysis, while those of the muscle compound and its deaminated derivative, inosinic acid, offer a much greater resistance [Levene and Jorpes, 1929]. The position of the phosphoric acid linkage in the muscle derivatives has been definitely established, namely to the terminal alcohol group of the ribose (C_5), while that of the similar linkage in the adenylic acid of yeast is probably the same as in guanylic acid [Levene and Harris, 1932] namely to C_3 of the ribose. The lability of the phosphoryl linkage in the yeast derivative is of much the same order as that of the two loosely bound

phosphoryl groups in adenosinetriphosphoric acid (a.t.p.) of skeletal muscle. The ease with which the labile linkages in the latter may be broken is shown in Embden's method of preparing adenylic acid from muscle, when under the action of $Ca(OH)_2$ at room temperature the original molecule loses two of the three phosphoric acid molecules. As will be seen later the nucleotide of cardiac muscle shows certain characteristic differences from that of skeletal muscle, due to the number and possibly also the position of the phosphoryl linkages. It is necessary always to bear in mind that the methods employed in separating the salts of these nucleotides may give rise to alterations in the character of the linkages. Fischer [1914] in his investigation of synthetic theophylline glucoside-phosphoric acid referred to the ease with which the phosphoryl linkage might be altered with reaction, thus the original monobasic acid with two of the OH groups of the phosphoric acid fixed to alcohol groups of the glucose might readily be changed to a dibasic acid with one only of the OH groups fixed to a single alcohol group.

In this communication special attention will be directed to the adenyl nucleotide in cardiac muscle, but it will be necessary to compare it with the closely allied substance met with in skeletal muscle. There are only two definite references, both very brief, to the adenyl derivative of heart muscle. One of these is by Lohmann [1931] in which the following characteristics of this body are just mentioned. The barium salt on neutral hydrolysis was found to furnish adenylic acid and pyrophosphoric acid, and the ratio of the loosely bound to the firmly bound phosphorus was found to be 1 : 1, instead of 2 : 1. The second reference occurs in a paper by Deuticke [1932] dealing with the action of the heart nucleotide on the uterus of the guinea-pig, in which he briefly mentions a statement made by Embden at a congress in Wiesbaden in 1932 to the effect that the substance is probably a dinucleotide consisting of one molecule of adenosinetriphosphoric acid and one of adenosinediphosphoric acid in which the ratio of N : P is therefore 10 : 5. Ostern and Mann [1933], dealing with the deamination process in heart-muscle, refer briefly to the statements made by Lohmann and Embden, but they did not themselves analyse the compound.

Evidence may be obtained very readily of the presence in heart-muscle of a substance possessing approximately two labile H_3PO_4 molecules to one firmly bound. Thus in the trichloroacetic acid extract of the muscle without separation of the substance, as also in the calcium hydroxide precipitate and in the mercuric acetate precipitate, the existence of such a substance is easily shown. When, however, further purification of the nucleotide is carried out in the way to be described, certain differences between the skeletal and cardiac derivatives become evident. The method of separation of the silver salt follows, generally speaking, the procedure originally adopted by Fiske and Subbarow for the skeletal muscle derivative, but their description is very brief.

Preparation of the silver salt of the cardiac nucleotide.

The same salt may be obtained from the hearts of the rabbit, cat, dog and ox, but in order to obtain larger quantities it is advisable to use the heart of the horse. The heart was removed immediately after death, the cavities being slit open and washed out with normal saline solution. The whole organ while still showing fibrillary contractions was placed in a large basin surrounded by freezing mixture and immediately taken to the laboratory for extraction. The more muscular parts were removed and minced. In this way about one kg. of muscle could be obtained, which was then extracted with two volumes of 5 % trichloroacetic acid, filtered, neutralised and again filtered. About two litres of

protein-free extract were obtained. Acetic acid was added up to 2 % and then saturated mercuric acetate solution in 2 % acetic acid until precipitation was complete. The deposit was separated by centrifuge and after washing with weak mercuric acetate solution the mercury was removed by H_2S and the latter by aeration. This solution was found to contain per g. muscle; total P, 0·029 mg.; P as orthophosphoric acid, none; P as orthophosphoric acid after hydrolysis for 10 mins. with *N* HCl at 100°, 0·02 mg. The solution was then concentrated *in vacuo* at 40° to a volume of approximately 150 ml. To this 20–30 ml. 20 % $CaCl_2$ and ethyl alcohol slightly acidified with HCl were added until precipitation was complete. The precipitate, after repeated solution and reprecipitation, was finally washed free from chlorine by 50 %, 70 % and finally absolute alcohol. After drying, the salt was dissolved in weak nitric acid and 10 % $AgNO_3$ added until the separation of the crystalline salt was complete. The precipitate was thoroughly washed with weaker alcohols and then with ethyl alcohol and ether and finally dried *in vacuo* at 80° over P_2O_5.

The yields from heart muscle were always much less than from skeletal muscle. The silver salt of the nucleotide in skeletal muscle was prepared in the same way.

Attention was directed especially to the estimation of the total nitrogen and phosphorus, the ribose and the silver.

Methods of analysis.

Nitrogen; by micro-Kjeldahl.

Phosphorus; by Fiske and Subbarow's method.

Ribose; the method employed was to determine the reducing value by Hagedorn and Jensen's method after half an hour's hydrolysis at 100° with *N* HCl to break the glucosidic linkage. Levene in his determinations of the pentose in the purine nucleotides found that the maximum reducing power was reached after about half an hour's acid hydrolysis and that the yield reached about 96 % of the theoretical value. He determined the reducing value of pure *d*-ribose and found it to be (by Hagedorn and Jensen's method) 87·5 % of the glucose value. Our calculations of the ribose value are based upon these data given by Levene. The furfuraldehyde yield was also determined and was always found to be higher using the method of Andrews and Milroy [1933] than with the Hoffman method.

Silver was estimated by the usual thiocyanate method. In all cases the silver salts analysed were free from inorganic phosphate. As will be seen the analyses of the silver salt of the skeletal muscle derivative correspond closely to a salt with the formula $C_{10}H_{13}O_{13}N_5P_3Ag_3$.

From Lohmann's electrotitration curves [1932] the acid appears to possess in its unbroken form four acid valencies, three primary and one secondary. In the silver salt two of the three silver atoms seem to be taken up by the primary acid groups in the labile diphosphoryl linkage and the other by the primary acid OH of the stable phosphoric acid linked to the terminal alcohol group in the ribose. If the acid be regarded, as appears probable, as tetrabasic with a labile anhydrodiphosphoryl linkage, the above formula includes one molecule of water.

	Calculated for $C_{10}H_{13}O_{13}N_5P_3Ag_3$ (827·84) %	Found	
		Spec. 1 %	Spec. 2 %
N	8·46	8·62	8·29
P	11·24	11·18	11·08
Ribose	18·12	18·00	17·78
Silver	39·09	39·12	38·85

The above are the analyses of the silver salts of two specimens of the skeletal derivative obtained from different sources.

The salt therefore corresponds to one with N : P = 5 : 3 and the agreement with the calculated values for silver and ribose is fairly close.

When the analyses of the cardiac derivative are examined such an agreement with an adenosinetriphosphate is no longer evident. For comparison with other possible nucleotide forms, the values found are to be compared not only with the silver salt of adenosinetriphosphate (I), but also with the theoretical values for a corresponding adenosinediphosphate (II) and for a dinucleotide consisting of an equimolecular mixture of these two (III).

	Calculated for			Found	
	I	II	III		
	$C_{10}H_{13}O_{13}N_5P_3Ag_3$ (827·84) %	$C_{10}H_{13}O_{10}N_5P_2Ag_2$ (640·94) %	$C_{20}H_{26}O_{23}N_{10}P_5Ag_5$ (1468·78) %	Spec. 1 %	Spec. 2 %
N	8·46	10·92	9·53	9·60	9·51
P	11·24	9·67	10·56	9·92	10·00
Ribose	18·12	23·40	20·43	19·80	19·50
Silver	39·09	33·66	36·72	34·82	35·40

Specimens I and II have N : P = 10 : 4·7 in place of 10 : 5 and the silver values are below the theoretical for such a dinucleotide. The salts are however much more closely allied to a mixture of one molecule of adenosinetriphosphate with one of the diphosphate than either of these in the pure form. That the labile phosphoryl linkages in the cardiac muscle derivative differ slightly from those in that of the skeletal muscle is evident from the results of hydrolysis with *N* HCl. Moreover one of the three labile phosphoric acid molecules may be set free on precipitation of the dinucleotide as a lead salt, giving then an adenosinediphosphate with a ratio of labile to stable P of 1 such as Lohmann observed. An example of this change having taken place may be given. A portion of the calcium salt of the cardiac derivative (0·2 g.) after removal of calcium was precipitated with normal lead acetate, the lead salt decomposed in the usual way and the solution made up to the same volume as before precipitation. The phosphorus distribution before and after precipitation by lead was as follows (mg. P per ml.).

	Before	After		
Orthophosphoric acid	nil	0·064	orthophosphoric	0·064
After 10 mins. acid hydrolysis	0·177	0·177	labile	0·113
Total	0·290	0·290	stable	0·113

In the solution prior to precipitation with lead 61 % of the total P was set free on acid hydrolysis (*N* HCl), corresponding therefore to 3 labile to 2 stable groups as in an equimolecular mixture of the tri- and di-phosphates. If the solution had contained the triphosphate alone, 66 % of the total P would have been in labile form.

Acid hydrolysis of the cardiac nucleotide.

In all such hydrolysis experiments with *N* HCl special attention requires to be directed to the amounts of inorganic phosphate set free within the first 10–30 mins., because, subsequent to the setting free of the labile P, the rate of hydrolysis of the stable P is very slow and for all such adenyl nucleotides as occur in muscle fairly constant.

The procedure adopted was as follows. One ml. of the solution containing a known quantity of the cardiac (or skeletal) derivative was mixed with 1 ml. of 2*N* HCl or 2*N* acetic acid and kept at 100° in sealed tubes for the periods stated.

After rapid cooling the solutions were neutralised and made up to a volume corresponding to the standard solution concentration (0·4 mg. P per 100 ml.), so that the colorimetric determinations might be carried out under strictly comparable conditions.

Table I. *Hydrolysis in* N *HCl.*

Skeletal muscle adenosinetriphosphoric acid.

Time (mins.)	(1) P as orthophosphate mg. per ml.	(2) % of total P	(3) % of labile P (2 in 3)
0	0		
10	0·137	67	100
15	0·137	67	
30	0·140	68	
60	0·140	71	
Total P	0·204		

Cardiac muscle dinucleotide.

			% of labile P (3 in 5)
0	0		
10	0·123	60	100
15	0·131	64·5	
30	0·137	67·4	
60	0·141	69·4	

Table II. *Hydrolysis in* N *acetic acid.*

Skeletal muscle adenosinetriphosphoric acid.

Time (mins.)	(1) P as orthophosphate mg. per ml.	(2) % of total P	(3) % of labile P
0	0		
10	0·020	9·8	14·7
15	0·030	14·7	22·0
30	0·046	22·5	33·8
60	0·073	35·2	53·6
120	0·125	61·2	91·8
210	0·136	67·0	100·0
Total P	0·204		Labile P 0·136

Cardiac dinucleotide.

0	0		
10	0·036	8·5	14·2
15	0·046	11·1	18·2
30	0·103	24·5	40·8
60	0·143	34·0	56·7
120	0·195	46·4	77·3
210	0·252	61·4	100·0
Total P	·420		Labile P 0·252

The skeletal nucleotide loses 2/3 of its P after 10 mins. hydrolysis with *N* HCl, while from the cardiac one under the same conditions 3/5 of the total P is set free. On hydrolysis with *N* acetic acid the same changes take place after boiling for

210 mins. After 120 mins. the proportion of the loosely bound P split off from the cardiac derivative is distinctly less than from the skeletal one.

Alkaline hydrolysis of the cardiac dinucleotide.

Lohmann and others have frequently referred to the liberation of orthophosphate or pyrophosphate from the breakdown of adenosinetriphosphoric acid by $Ca(OH)_2$ and $Ba(OH)_2$. It is often impossible to be certain from statements in the literature whether the precipitates of so-called calcium or barium pyrophosphates were purely inorganic or not.

Precipitates obtained after half an hour's hydrolysis in $Ca(OH)_2$ always contain a fairly high percentage of N, although the main product, adenylic acid, forms a soluble calcium salt.

Mixtures of one volume of the solutions of the two nucleotides with one volume of saturated $Ca(OH)_2$ were kept at 100° in sealed tubes for the periods stated, and the orthophosphate at each stage was determined. The mixtures were shaken throughout.

The percentage of labile P in the adenosinetriphosphoric acid is calculated as 66 % of the total, that of the dinucleotide as 60 %.

Table III.

	Skeletal adenosinetriphosphoric acid			Cardiac dinucleotide		
	1	2	3	1	2	3
Time (mins.)	P as ortho-phosphate mg. per ml.	% of total P	% of labile P	P as ortho-phosphate mg. per ml.	% of total P	% of labile P
0	0					
5	0·029	30·2	45·3	0·043	43·0	71·6
10	0·047	48·9	73·4	0·045	45·0	75·0
15	0·047	48·9	73·4	0·047	47·0	78·3
30	0·049	51·0	76·5	0·047	47·0	78·3
90	0·066	68·7	100·0	0·050	50·0	83·3
120	0·066	68·7	100·0	0·064	64·0	100·0
180	—	—	—	0·064	64·0	—
	Total P	0·096			Total P	0·100

Difficulties arise in the determination of the rates of hydrolysis with $Ca(OH)_2$ owing mainly to the fact that the salts to be acted upon are not in a state of solution but in one of fine suspension. After the loosely bound phosphoric acid molecules have been set free, the calcium salt of adenylic acid along with varying amounts of adenosine should pass into solution. As it is the amount of ortho-

Table IV.

Time (mins.)	P as orthophosphate mg. per ml.	%
0	0	
5	0·077	48·1
10	0·102	63·6
15	0·130	81·2
30	0·130	81·2
90	0·160	100·0
120	0·160	
	Total P 0·160	

phosphate which is being estimated at the different stages, the rate of hydrolysis of the inorganic pyrophosphate by $Ca(OH)_2$ requires to be known as the loosely bound phosphorus in the nucleotides may be set free in this form. Table IV gives the results of the hydrolysis of sodium pyrophosphate with $Ca(OH)_2$.

When the results of the hydrolysis of the three compounds are examined, it is evident that one factor which may determine the rate of orthophosphate formation from the organic substances is the rate of change of pyrophosphate into orthophosphate. That this is not the only factor in the case of the dinucleotide is evident from the greater initial rate at which orthophosphate is set free from it compared with inorganic pyrophosphate. The labile portion of adenosinetriphosphoric acid behaves somewhat similarly to the inorganic salt on alkaline hydrolysis, just as it does in the case of hydrolysis with *N* HCl. At present no definite evidence is obtainable regarding the position of the labile phosphoric acid molecules in these nucleotides, but it appears probable that there is an anhydrodiphosphoryl linkage in the triphosphoric acid which may combine in the case of dinucleotides with a secondary alcohol group of the ribose in either an adenosinemonophosphate or diphosphate.

II. THE BIOLOGICAL AND CO-FERMENT PROPERTIES OF THE CARDIAC DINUCLEOTIDE.

THE definite biological actions of the adenosinetriphosphoric acid of skeletal muscle origin and the function of that nucleotide in lactic acid production suggest that an investigation into the pharmacology and co-ferment properties of the cardiac dinucleotide would be of special interest for several reasons. In the first place the substance is a natural component of the heart-muscle, and as such its actions on the heart and vessels are of importance, and in the second place, as chemically it presents certain characteristic differences from the derivative of skeletal muscle, any difference in action between the two might be explicable from the standpoint of chemical constitution. Finally, as proprietary preparations of tissue nucleotides and nucleosides are in routine use in the treatment of cardio-vascular disease, any further investigation into the pharmacology of such substances is of value.

Drury and Szent-Györgyi [1929] have studied the biological actions of a cardiac derivative which, as they deduced from careful analyses, was undoubtedly adenylic acid. They refer only briefly to the action of Fiske's adenosinetriphosphate (probably from skeletal muscle).

In all the following experiments the calcium salt of the cardiac dinucleotide was used.

The results are exactly comparable with those of Gillespie [1933] for the skeletal muscle nucleotide, in most cases both salts being tried on the same preparation.

Biological actions.

1. *The heart.* (*a*) Doses of 1 in 50,000 of the cardiac nucleotide perfused through the isolated frog's heart cause no alteration in the normal beat. Doses of 1 in 25,000 produce an A.V. block which passes off on perfusion with fresh Ringer's solution.

(*b*) In the isolated perfused rabbit's heart, doses of 0·1 to 0·2 mg. of the heart dinucleotide added to the perfusion fluid cause first a diminution in rate and amplitude of the beat followed in a few seconds by augmentation of the beat and an increased coronary outflow.

2. *Blood pressure.* Doses as small as 0·01 mg. lower the carotid arterial pressure of the rabbit. The fall in pressure is not so sudden as that produced by vagal stimulation. The fall is preceded by a short rise. The whole effect is quite transitory, and complete recovery takes place in a few seconds. This was also seen after atropine.

3. *Perfusion of vessels.* Frogs' vessels were perfused in the ordinary way. Doses of 0·2 mg. injected at the point of entrance of the perfusion fluid doubled the drop rate in about 1½ mins.

4. *Uterus.* Concentrations of 1 in 250,000 of the cardiac dinucleotide increase the tone of the virgin guinea-pig's uterus perfused by the Sawasaki technique and finally give rise to spontaneous contractions. This is the same effect as is described by Deuticke [1932] in connection with Embden's heart nucleotide.

5. *Intestine.* Concentrations of 1 in 50,000 and less lower the tone of the perfused small intestine of the rabbit. This is a different effect from that produced by the voluntary muscle nucleotide, which causes a marked increase in tone immediately after the fall [Gillespie, 1933]. Using the intestine as a test object it was shown that dialysates of saline extracts of heart muscle owe their biological action to the dinucleotide component.

The action of the cardiac dinucleotide on the amphibian and mammalian heart and vessels and on the perfused uterus closely resembles, both qualitatively and quantitatively, the action of the voluntary muscle nucleotide as described by Gillespie. Voluntary muscle nucleotide produces a fall in intestinal tone followed by a marked rise. Only the fall has been observed with the cardiac derivative.

Ferment actions of the cardiac nucleotide.

The glycolytic ferment of voluntary muscle consists of two fractions—the colloidal ferment and a co-ferment which Meyerhof and Lohmann [1928] have identified with adenylpyrophosphate. Little is known of the glycolytic ferment system in the heart, and as the dinucleotide just described has been tested as co-ferment on cardiac extracts acting on artificial substrates of starch or glucose with orthophosphate, the results of this investigation help to complete the comparison of the two types of nucleotide. Lambs' hearts fresh from the abattoir were used for these experiments.

The first experiments show that an active ferment system can be extracted from the mammalian heart muscle with ice-cold saline. This activity is shown by the appearance of lactic acid in the incubation mixture containing starch and inorganic phosphate and by the disappearance of some of the orthophosphate due to the synthesis of esters. For example:

2nd and 3rd saline extracts incubated 2 hrs. at 25° with starch substrate.

m.mols ester produced	5·6
m.mols lactic acid produced	5·0

When the extract has been kept for 24 hrs. at 4° it no longer produces these effects. The addition of a small amount of the cardiac dinucleotide, or the

adenosinetriphosphoric acid of skeletal muscle, restores the activity of the extract. The following experiment will illustrate the type of action.

2nd and 3rd saline extracts, kept 24 hrs. at 4°, incubated 3½ hrs. at 25° with starch substrate.

1. Extract + substrate + no addition.
   m.mols ester produced ... ... 0·39
   m.mols lactic acid produced ... ... nil
2. Extract + substrate + 0·1 mg. skeletal adenosinetriphosphoric acid per ml.
   m.mols ester produced ... ... 0·70
   m.mols lactic acid produced ... ... 0·79
3. Extract + substrate + 0·1 mg. cardiac dinucleotide per ml.
   m.mols ester produced ... ... 2·10
   m.mols lactic acid produced ... ... 2·00

The cardiac dinucleotide reactivates the extract to a greater degree than does the nucleotide of voluntary muscle. It was found that this occurred also in the case of the reactivation of voluntary muscle extracts. Haarman [1932], in a paper published while these experiments were being carried out, points out that the preferential substrate for heart glycolysis is glucose. Again using the extract described above, but with glucose in place of starch as substrate, the results obtained were as follows:

1. Extract + substrate (glucose) + no addition.
   m.mols ester produced ... ... 0·6
   m.mols lactic acid produced ... ... 0·1
2. Extract + substrate (glucose) + 0·1 mg. skeletal muscle adenosinetriphosphoric acid per ml.
   m.mols ester produced ... ... 0·7
   m.mols lactic acid produced ... ... 1·0
3. Extract + substrate (glucose) + 0·1 mg. cardiac dinucleotide per ml.
   m.mols ester produced ... ... 0·0
   m.mols lactic acid produced ... ... 2·0

In these and other examples it was seen that the extract did not show a preference for glucose, though the observation was confirmed that the heart could use this substrate. It is evident, however, that while esterification is a feature of lactic acid production from starch, this is not the case when the substrate is glucose. Traces of ester may be found with glucose as substrate due to the glycogen content of the original extract. When minced heart muscle is added to an artificial substrate, it is found that, while starch can be used, there is a definite preference for glucose. As in the case of the saline extracts there is no tendency for esters to accumulate when the glucose substrate is used:

3 g. specimens of minced muscle were allowed to act on the phosphate, glucose and starch substrates in a total volume of 20 ml. for 2½ hrs. at 25°.

1. Glucose substrate.
   Orthophosphate used up ... ... 0·52 mg. P in 20 ml.
   Lactic acid produced ... ... 1·92 mg. in 20 ml.
2. Starch substrate.
   Orthophosphate used up ... ... 2·14 mg. P in 20 ml.
   Lactic acid produced ... ... 0·60 mg. in 20 ml.

It also has been found that a more powerful ferment system can be extracted from muscle which has been autolysed for 2 hours than from fresh muscle. The extract of autolysed muscle can be made still more active by addition of co-ferment. It is evident that during autolysis the colloidal component of the ferment system undergoes some change which facilitates its extraction.

The ferment system of cardiac muscle is, on the whole, not nearly so active as the corresponding system in voluntary muscle.

That glucose should be available as an immediate source of energy for the heart, whether or not glycogen is present, is of great interest. The glycolytic ferment system of voluntary muscle is incapable of using glucose directly. This means that, apart from the smooth muscle tissues with their slow rate of metabolism, the heart has an option on sugars in the circulation, a mechanism which must be of considerable importance in maintaining the heart beat in cachexia and privation when glycogen is low. It may also explain in part the therapeutic value of parenteral glucose saline in the treatment of shock and allied conditions.

Summary.

1. The nucleotide of cardiac muscle differs chemically from the adenosinetriphosphoric acid of voluntary muscle, being more closely allied to a dinucleotide composed of one molecule of adenosinediphosphoric acid and one molecule of adenosinetriphosphoric acid.

2. This is shown by (*a*) its chemical composition and (*b*) its behaviour on hydrolysis at 100° with *N* HCl. Approximately 60 % of its total P is in the labile form in place of 66–67 % in adenosinetriphosphoric acid.

3. The pharmacological actions are very similar to those of the voluntary muscle derivative, the only distinctive difference being observed in the reaction of the intestinal musculature, the dinucleotide lowering the tone without a subsequent rise, while adenosinetriphosphoric acid has the double action.

4. The heart dinucleotide has a more powerful co-ferment action as regards the reactivation of inactive ferment extracts both of voluntary and cardiac muscle.

REFERENCES.

Andrews and Milroy (1933). *Biochem. J.* **27**, 1421.
Barrenscheen and Filz (1932). *Biochem. Z.* **250**, 281.
Deuticke (1932). *Pfluger's Arch.* **230**, 539.
Drury and Szent-Györgyi (1929). *J. Physiol.* **68**, 213.
Fischer (1914). *Ber. deutsch. chem. Ges.* **47**, 3193.
Fiske and Subbarow (1929). *Science*, **70**, 382.
Gillespie (1933). *J. Physiol.* (in press).
Haarman (1932). *Biochem. Z.* **255**, 104.
Levene and Harris (1932). *J. Biol. Chem.* **95**, 155.
—— and Jorpes (1929). *J. Biol. Chem.* **81**, 575.
Lohmann (1929). *Naturwiss.* **17**, 624.
—— (1931). *Biochem. Z.* **233**, 467.
—— (1932). *Biochem. Z.* **254**, 391.
Meyerhof and Lohmann (1928). *Biochem. Z.* **196**, 22, 49.
Ostern and Mann (1933). *Biochem. Z.* **260**, 353.

XVI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXXVI. THE METABOLIC PRODUCTS OF *PENICILLIUM CHARLESII* G. SMITH.

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During an examination of a sample of mouldy Italian maize our late colleague, Mr J. H. V. Charles, isolated a new species of *Penicillium* belonging to Thom's group "The Monoverticillata-Ramigena" [Thom, 1930]. A description of this mould, which has been named *P. Charlesii*, has been published elsewhere [Smith, 1933]. When grown on Czapek-Dox solution or on the medium which we have called Raulin-Thom solution [Clutterbuck *et al.* 1932] the culture medium becomes very strongly acid, reduces $KMnO_4$ instantly in the cold, gives an intense orange or scarlet colour with ferric chloride, and on addition of excess of alcohol gives a voluminous sticky precipitate. In addition, if the period of incubation is sufficiently prolonged, the metabolism solution becomes definitely laevorotatory.

Addition of acetone to the evaporated metabolism solution precipitated a mixture of two new polysaccharides, one of which was a polygalactose and the other a polymannose. The syrupy precipitate obtained with acetone both from Czapek-Dox solution and Raulin-Thom solution gave, on trituration with absolute alcohol and ether, an amorphous hygroscopic powder with a high content of ash consisting chiefly of phosphates. Most of this ash, however, could be removed by precipitation with alcohol.

The aqueous solution of the crude product from Raulin-Thom medium was acid to litmus, with an average rotation of $[\alpha]_{5790} = -40°$, and had no action on Fehling's solution or on iodine. Its behaviour on hydrolysis suggested the presence of two polysaccharides. Treated at 100° with $0{\cdot}4N$ sulphuric acid the rotation changed from $-40°$ to $+61°$, but on pouring the equilibrium solution into alcohol a considerable portion of a polysaccharide was precipitated. With $3N$ sulphuric acid, however, this precipitated portion hydrolysed readily, the rotation changing from $+50°$ to $+14{\cdot}3°$, the latter value being identical with the equilibrium rotation of *d*-mannose. The crystalline hydrolysis product from the more labile polysaccharide was identified as *d*-galactose, and that from the more stable polysaccharide was identified as *d*-mannose. By a very tedious process of fractional precipitation with alcohol a sample of the polymannose $[\alpha]_{5790} + 63°$ in water, and a sample of the polygalactose $[\alpha]_{5790} = -84°$ in water were obtained, thus showing that the original product was a mixture. The crude polymannose was a white hygroscopic powder containing approximately 15 %

of moisture and 10 % of ash, most of the latter being removed by precipitation from hydrochloric acid solution with alcohol. It gave a colourless acid solution in water and did not react with iodine or Fehling's solution. Hydrolysis with 3*N* sulphuric acid gave only mannose. On acetylation a homogeneous triacetyl compound was obtained from which the original polymannose could be regenerated with sodium hydroxide. The polygalactose was identical in appearance with the polymannose. It contained only 1·8 % of ash when crude and approximately 10 % of moisture. It was readily hydrolysed by even dilute acid to give only galactose. An ash-free triacetyl derivative was prepared which could not, however, be satisfactorily deacetylated with sodium hydroxide. The aqueous solution of the polygalactose was colourless, acid to litmus and had no action on iodine or Fehling's solution.

From Czapek-Dox medium a mixture of the same two polysaccharides was obtained. This crude mixture had a very high ash content and contained a larger proportion of the polymannose.

After precipitation of the polysaccharides with acetone, the acetone mother-liquors were evaporated *in vacuo* to remove acetone, acidified with HCl and exhaustively extracted first with ether and then with chloroform. These solvents extract a mixture of at least six new optically active acids, which have been shown to have the following empirical formulae, and for which we propose the accompanying names: $C_9H_{10}O_4$, *carolic acid*; $C_9H_{12}O_7$, *carolinic acid*; $C_{10}H_{10}O_6$, *carlic acid*; $C_{10}H_{12}O_6$, *carlosic acid*; $C_{16}H_{20}O_6$, *ramigenic acid*; $C_{26}H_{32}O_{12}$, *verticillic acid*.

Carolic acid, $C_9H_{10}O_4$, is produced in the largest yields and occurs principally in the chloroform extracts and to a much smaller extent in the ether extracts. It is produced on both media, though in larger yield on the Czapek-Dox medium. It is a dextrorotatory monobasic acid and gives a characteristic orange-yellow coloration with $FeCl_3$.

Carolinic acid, $C_9H_{12}O_7$, is the acid produced in the next largest yield to carolic acid and occurs principally in the ether extracts. The largest yields of this acid were obtained from Raulin-Thom's medium. It is a dextrorotatory dibasic acid and gives a characteristic copper salt which crystallises in pure blue needles.

Carlic acid, $C_{10}H_{10}O_6$, is produced in small amounts on both media and is present almost entirely in the chloroform extracts. It is a laevorotatory dibasic acid.

Carlosic acid, $C_{10}H_{12}O_6$, a laevorotatory dibasic acid, was obtained only on Czapek-Dox medium, none being isolated from Raulin-Thom medium. It occurs entirely in the ether extracts and was obtained in moderate yields. Carlosic acid is differentiated from the other metabolic products of *P. Charlesii* by the heavy brownish-yellow precipitate with $FeCl_3$, insoluble in excess of this reagent, by the formation on standing of a crystalline yellow precipitate with uranium acetate and by the immediate heavy precipitate, soluble in excess, which is given on careful addition of normal lead acetate.

Ramigenic acid, $C_{16}H_{20}O_6$, a dextrorotatory acid, was obtained in small yields on both media, occurring entirely in the ether extracts.

Verticillic acid, $C_{26}H_{32}O_{12}$, a laevorotatory tetrabasic acid, was obtained on both media, the larger yields being given on Raulin-Thom medium. It occurs almost entirely in the ether extracts, only traces being isolated from the chloroform extracts. A neutral aqueous solution of its sodium salt reduces silver nitrate in the cold, gives a heavy precipitate with mercuric chloride from which it can be regenerated unchanged, a definite orange-yellow colour with uranium acetate and a very intense reddish brown colour with $FeCl_3$.

In addition to the products described above, a certain amount of non-crystalline syrup has been separated. Moreover, the aqueous phase after exhaustive solvent extraction is still laevorotatory. We propose to investigate both of these fractions further.

It is the purpose of this paper to describe the cultural methods employed for the production of the substances described above, the chemical methods used for their isolation and purification and their general properties. The question of their molecular constitution will be dealt with in a series of subsequent papers.

Experimental.

Organism and culture media.

The organism used during the whole of this work was the new species of *Penicillium, P. Charlesii* G. Smith (London School of Hygiene Catalogue Number P 146). (*Cf.* Smith, 1933.)

Preliminary experiments in test-tubes showed that the reactions characteristic of *P. Charlesii* are given when the mould is grown on either the well-known Czapek-Dox solution or on the modified Raulin's solution which we have called the Raulin-Thom medium. The compositions of the two media are the following:

Czapek-Dox solution. Water, to 1000 ml.; glucose, 50 g.; $NaNO_3$, 2 g.; KH_2PO_4, 1 g.; KCl, 0·5 g.; $MgSO_4, 7H_2O$, 0·5 g.; $FeSO_4, 7H_2O$, 0·01 g.

Raulin-Thom solution. Water, to 1500 ml.; glucose, 75 g.; tartaric acid, 4 g.; ammonium tartrate, 4 g.; diammonium hydrogen phosphate, 0·6 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $(NH_4)_2SO_4$, 0·25 g.; $ZnSO_4, 7H_2O$, 0·07 g.; $FeSO_4, 7H_2O$, 0·07 g.

The course of metabolism.

A number of one litre conical flasks, each containing 350 ml. of Czapek-Dox solution, and a similar number of flasks of Raulin-Thom solution were sterilised and sown with a spore-suspension of *P. Charlesii* and incubated at 24°. Single flasks were taken at intervals for examination of the metabolism solution.

On Czapek-Dox solution growth was rapid but the mycelial felt was thin, the surface being rich dark green in colour and with a white reverse. The solution became very tardily pink (21 days), then brownish yellow. The rate of metabolism, as shown by analysis of the contents of single flasks, was somewhat erratic. Representative analyses are given in Table I in which are shown the

Table I.

Days of incubation	p_H	Rotation Hg_{5461} in 2 dm. tube	Apparent residual glucose (%)	$FeCl_3$ reaction
0	4·5	+6·25°	5·04	Nil
3	5·1	+6·18	4·98	Nil
6	4·8	+5·72	4·61	Pale yellow
9	4·7	+5·42	4·37	Yellow
17	4·6	+4·35	3·51	Very deep yellow
25	3·4	+3·05	2·46	Orange-yellow
32	3·1	+1·73	1·40	Deep orange
51	2·4	−0·39	—	Orange-red

apparent rate of disappearance of the sugar, as determined by polarimeter (these figures are obviously somewhat inaccurate owing to the presence of optically active metabolic products), the change of reaction of the medium and the colours

obtained on addition to 5 ml. of the solution of a few drops of ferric chloride solution. An attempt was made to estimate bromine absorption by Koppeschaar's method [1876], but although it was apparent in the case of the older solutions that very considerable absorption took place, back-titration was impossible owing to rapid decomposition of the bromine compounds.

On Raulin-Thom solution growth was more rapid and the course of metabolism less erratic than on Czapek-Dox solution. The mycelial felts were comparatively thick, being bright green on the surface with a reverse which became rapidly mauve and then brownish in colour, the solution being first bright rose-pink and then brownish yellow in colour. In view of the failure to obtain satisfactory results for the estimation of bromine absorption by the Koppeschaar method an attempt was made to obtain some measure of the reactive substances produced by the mould by estimating the amount of $KMnO_4$ reduced under carefully standardised conditions. The results obtained, however, were unsatisfactory. The results obtained on the Raulin-Thom medium are given in Table II.

Table II.

Days of incubation	p_H	Rotation Hg_{5461} in 2 dm. tube	Apparent residual glucose (%)	$FeCl_3$ reaction
0	3·9	+6·61°	5·34	Bright yellow
3	3·6	+6·07	4·90	Bright yellow
6	2·5	+3·64	2·94	Deep golden yellow
9	2·3	+1·90	1·53	Bright orange
11	2·2	+1·22	0·98	Bright orange
14	2·0	+0·70	0·56	Bright orange
17	2·0	+0·30	0·24	Deep orange
20	2·0	+0·11	0·09	Reddish orange
23	2·2	0	0	Reddish orange

Cultivation of P. Charlesii *in the routine metabolism experiments.*

In each of twelve experiments 100 one litre conical flasks, each containing 350 ml. of the selected medium, were sterilised by steaming on three successive days and were then sown with an aqueous spore suspension made from four cultures, 2 to 3 weeks old, grown on beer wort-agar slopes. The flasks were incubated at 24° until the sugar had completely disappeared, as evidenced by the solution becoming optically inactive or slightly laevorotatory. The duration of incubation on the Raulin-Thom medium was 24–28 days and on the Czapek-Dox medium about 8 weeks.

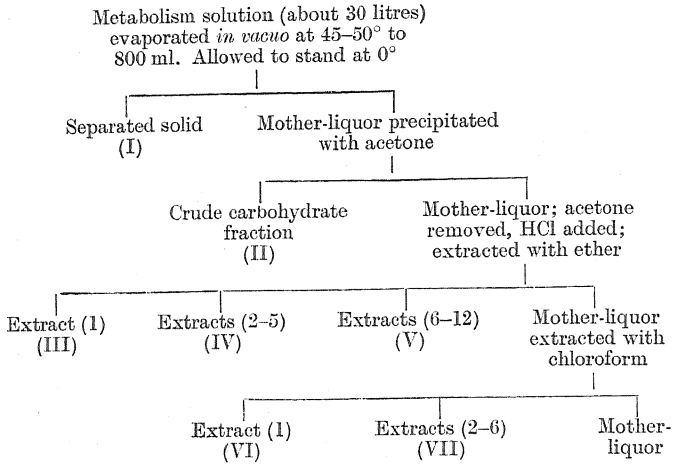
Separation of metabolic products.

At the end of the incubation period the metabolism solution was filtered from the mycelium, and the latter was well pressed and washed with a small quantity of water, the washings being added to the main filtrate. The metabolic products were first separated from the metabolism solution in a series of fractions which were eventually examined individually. The general scheme of separation of these fractions was as shown on p. 98.

This scheme relates to the experiments with Raulin-Thom medium. With Czapek-Dox medium the method of separation employed was the same but the grouping of the extracts was slightly different. Thus for Czapek-Dox medium, fraction (IV) refers to ether extracts (2–3), fraction (V) to ether extracts (4–10) and fraction (VII) to chloroform extracts (2–5).

The combined filtered metabolism solution and washings from 100 flasks were evaporated *in vacuo* at 45–50°, using the apparatus described by Raistrick and

Smith [1933], to 800 ml. and allowed to stand for 1 day at 0°. An amorphous solid separated and was filtered off, washed and dried (fraction I). To the mother-liquor, now about 1000 ml. in volume, were added 4 litres of commercial acetone, the mixture being well stirred and allowed to stand until the sticky



precipitate produced had completely settled. The clear liquid was decanted off and the precipitate washed twice with small quantities of acetone, about 200 ml. in all. The residual sticky solid was dissolved in 200 ml. of water, the solution filtered and the crude carbohydrate reprecipitated in a less sticky form by the rapid addition of 9 volumes of ethyl alcohol. The solid was separated by decantation, then ground with absolute ethyl alcohol until the whole was in the form of a powder, which was filtered off, well drained at the pump and dried in air (fraction II).

The combined aqueous acetone mother-liquors and washings were evaporated *in vacuo* to about 600 ml. in order to remove all the acetone. To the concentrate, 100 ml. concentrated HCl were added. A small amount of oily material separated at this stage but was ignored as it was difficult to separate and was readily redissolved during the subsequent extractions. The acidified solution was extracted 12 times (Raulin-Thom) or 10 times (Czapek-Dox) with its own volume of ether. Using Raulin-Thom medium, the first extract (fraction III) was kept separate and extracts 2–5 were combined as fraction IV, as were also extracts 6–12 as fraction V. With Czapek-Dox medium extracts 2–3 were combined as fraction IV and extracts 4–10 were combined as fraction V. The aqueous mother-liquor was then extracted 6 times (Raulin-Thom) or 5 times (Czapek-Dox) with its own volume of chloroform, the first extract (fraction VI) being kept separate and the remaining extracts being combined as fraction VII. All the extracts were evaporated *in vacuo* to give orange syrups which on keeping for some time gradually solidified to sticky masses of small crystals.

The aqueous solution, after extraction with chloroform, still gave a faint orange colour with ferric chloride, readily reduced $KMnO_4$ and was definitely laevorotatory. Extraction with ether or with chloroform in a continuous extractor for several days removed only very small amounts of dark-coloured sticky syrup. It is possible that the residual unextracted acid is carlic acid $C_{10}H_{10}O_6$, since its relative solubility in water, ether and chloroform would render its extraction very slow (see p. 106).

The dry weights of all the above fractions for eight typical experiments (Nos. 5 to 12), four on each medium, are summarised in Table III. The fractions from Exp. 1–4 (twelve experiments in all, six on each medium, have been carried out) were used in the preliminary work of detecting and isolating the various products present, of obtaining their solubilities and investigating their general properties.

Table III.

Exp. No.	Medium	Fractions: weight in g. I	II	III	IV	V	VI	VII
5	Czapek-Dox	10·9	53·6	30·0	35·4	40·2	51·8	25·6
6	,,	25·4	48·7	30·3	18·1	30·2	50·0	15·7
7	,,	5·9	51·6	30·0	25·6	36·0	44·0	20·5
10	,,	11·5	45·6	22·0	14·2	20·8	40·2	20·8
8	Raulin-Thom	4·0	39·7	18·0	37·9	26·8	30·4	13·6
9	,,	5·4	41·0	29·0	31·0	21·4	26·0	12·3
11	,,	4·9	43·0	19·4	26·0	19·0	24·8	11·2
12	,,	1·7	44·8	31·4	27·0	18·0	24·0	14·0

Since in each experiment 100 flasks were used containing in all 1750 g. glucose at the beginning of the experiment, and the flasks were taken off when the sugar had disappeared, the yields of these fractions in relation to the sugar utilised can readily be computed.

Isolation of products from fractions I–VII.

A. *Fraction I.*

This fraction consisted, using both media, of a yellowish-brown, amorphous powder which was insoluble in all the usual organic solvents, was non-acidic, contained no nitrogen and did not melt on heating but charred at about 250°. It was not further investigated.

B. *Fraction II.*

The following are the details of the preliminary examination of this fraction, which consisted essentially of a mixture of two polysaccharides together with mineral salts. A more detailed investigation of these polysaccharides is now in progress and will be reported on in a subsequent paper.

I. *Investigation of the polysaccharides from Raulin-Thom's medium.* The crude polysaccharide was precipitated as a red viscid mass by the addition of excess acetone to the concentrated metabolism solution. On trituration with absolute alcohol and washing with ether it was obtained as a yellowish hygroscopic powder which tended to form a glutinous mass on exposure to the atmosphere. The four batches of the material which were prepared had the following properties.

Table IV.

Exp. No.	Yield in g.	% ash content	$[\alpha]_{5790}$ in water
8	39·7	9·5	$-41·5°$ ($c=2·31$)
9	41·0	10·3	$-39·5°$ ($c=2·17$)
11	43·0	4·0	$-38·7°$ ($c=2·07$)
12	44·8	5·7	$-40·1°$ ($c=2·68$)

The polysaccharide readily dissolved in water to give a milky yellow solution which rapidly cleared on centrifuging. It had no action on Fehling's solution or

on iodine and was acid to litmus. No pentose could be detected by the furfuraldehyde test but the solution after treatment with acid reduced Fehling's solution in the cold.

Hydrolysis of the crude polysaccharide with 0·4N H_2SO_4. A portion of the crude polysaccharide from experiment 8 containing 9·5 % ash was dissolved in 0·4*N* H_2SO_4, heated on the boiling water-bath and the rotation followed polarimetrically ($c=1·16$) with the following results.

0 minutes $[\alpha]_{5790}=-41·5°$, 5 minutes $[\alpha]_{5790}=-20·1°$, 10 minutes $[\alpha]_{5790}=0°$, 15 minutes $[\alpha]_{5790}=+27·2°$, 45 minutes $[\alpha]_{5790}=+55·9°$, 90 minutes $[\alpha]_{5790}=+60·5°$ (equilibrium value). This equilibrium value, which on correction for ash $=66·2°$, gave no definite indication of the sugar formed, and it was found that on pouring the rotation solution into absolute alcohol a polysaccharide was precipitated. This precipitate was removed by centrifuging, washed with absolute alcohol and ether and dried *in vacuo*. It was a white amorphous powder, soluble in water with an acid reaction to litmus and contained approximately 10 % of ash.

It was stable at 100° to 0·4*N* H_2SO_4 for 3 hours but was hydrolysed as follows by 3*N* H_2SO_4 at 100° ($c=0·97$ and rotation corrected for ash). 0 minutes $[\alpha]_{5790}=+48·7°$, 10 minutes $[\alpha]_{5790}=+29·9°$, 30 minutes $[\alpha]_{5790}=+15·5°$, 60 minutes $[\alpha]_{5790}=+14·3°$ (equilibrium value). This equilibrium value is very close to that of *d*-mannose $[\alpha]^{20}_{5790}=+15·2°$.

The crude polysaccharide (3·5 g.) was dissolved in 0·4*N* H_2SO_4 (50 ml.) and heated for 90 minutes until the rotation was constant. Alcohol was added to the cold solution until there was no further precipitate. This precipitate was isolated by the method described above. Yield $=1·1$ g. Ash content $=10·1$ %.

Examination of the alcoholic mother-liquors. A little water was added and the sulphuric acid neutralised with barium carbonate. The barium sulphate was filtered off and the solution concentrated *in vacuo* at 30°. The syrupy product was extracted with hot 95 % ethyl alcohol until the residue showed no reducing properties. Concentration of the alcoholic extract gave a clear colourless syrup which crystallised on scratching. Yield, 1·9 g.; M.P. crude 160°. Recrystallised from aqueous alcohol the crystals had M.P. 165° alone or in admixture with *d*-galactose; $[\alpha]_{5790}=148·7°\rightarrow 83·8°$ ($c=1·11$) after 2 days (equilibrium value for *d*-galactose $[\alpha]^{20}_{5790}=+83·6°$). The identity of the galactose was confirmed by the formation of galactosazone (M.P. and mixed M.P. 196°) and by the preparation, on oxidation with nitric acid, of *d*-mucic acid (M.P. 214°); yield, 77 % theoretical. No other sugar could be detected.

Hydrolysis of the more stable polysaccharide. This polysaccharide (1·1 g.) was heated for 90 minutes at 100° with 50 ml. of 3*N* sulphuric acid. The acid was neutralised with barium carbonate, the barium sulphate filtered off and the solution concentrated *in vacuo* at 30°. The syrup was extracted with 95 % alcohol, the removal of which by evaporation gave a colourless product.

On treatment of a portion of the syrup (0·2 g.) with phenylhydrazine acetate (0·2 g.) in the cold for ½ hour a sandy crystalline powder separated. After standing for an hour the crystals were filtered off and recrystallised from 50 % alcohol (0·28 g.), from which solvent they separated in the characteristic form of mannosephenylhydrazone and had M.P. and mixed M.P. with an authentic specimen 196°.

This phenylhydrazone, on heating with an excess of phenylhydrazine acetate at 100° for 5 minutes, yielded glucosazone identical with an authentic specimen.

The remainder of the syrup was scratched with a nucleus of *d*-mannose and crystallised completely in a few days, M.P. 135° (M.P. of mannose 132–133°).

Fractionation of the mixture of the two polysaccharides. It was found that the crude polysaccharide could be separated into various fractions by the graded addition of absolute alcohol to a concentrated aqueous solution. In a typical experiment 78 g. of crude polysaccharide $[\alpha]_{5790} = -41{\cdot}0°$ gave the following results.

Table V.

Fraction	$[\alpha]_{5790}$	% alcohol	% ash	Yield (g.)
1	0	50	40·3	12
2	−34·2°	66	3·5	40
3	−37·6°	80	5·8	9
4	−56·7°	Concentrated mother-liquor	3·7	14

Most of the ash was removed in the first fraction and it was found that the ash could be further separated as follows. 43 g. of crude polysaccharide with a high ash content were dissolved in 150 ml. of water. To this syrup, pyridine (200 ml.) was carefully added. A fraction (7 g.) was precipitated and removed by centrifuging. This fraction contained 90 % of ash. The remaining fractions could be precipitated by the addition of alcohol to the aqueous pyridine solution.

By an elaborate and tedious method of repeated precipitations it was possible to isolate specimens of a polymannose having $[\alpha]_{5790} = +63°$ (15 g.) and a polygalactose $[\alpha]_{5790} = -84°$ (12 g.). A series of intermediate fractions is in process of separation.

Properties of the purified polymannose. This compound was a pure white hygroscopic powder containing, before final purification, 15 % of moisture and 10 % of ash. The ash content was lowered to 2·5 % after one precipitation from weak hydrochloric acid solution by the addition of alcohol. Its aqueous solution was colourless, acid to litmus, non-reducing and without action on iodine. It was stable to N H_2SO_4 at 80° for 2 hours but was readily hydrolysed with $3N$ H_2SO_4 at 100°. A sample having an ash content of 0·7 % and dried to constant weight gave the following results on hydrolysis with $3N$ H_2SO_4 at 100°:

0 minutes $[\alpha]_{5790} = +63{\cdot}0°$, 10 minutes $[\alpha]_{5790} = +49{\cdot}1°$, 25 minutes $[\alpha]_{5790} = +31{\cdot}4°$, 45 minutes $[\alpha]_{5790} = +17{\cdot}7°$, 60 minutes $[\alpha]_{5790} = +15{\cdot}7°$, 90 minutes $[\alpha]_{5790} = +15{\cdot}7°$. Therefore the equilibrium rotation calculated as hexose $= +14{\cdot}1°$ (equilibrium rotation recorded for mannose $= +15{\cdot}2°$). Mannose was the only hydrolysis product isolated.

An analysis was carried out on the incompletely dried polysaccharide and the moisture content estimated separately. The analysis was not entirely satisfactory owing to the hygroscopic nature of the ash.

(Found: Moisture, 15·0 %; ash, 9·6 %; C, 44·6; H, 7·5 %. $(C_6H_{10}O_5)_n$ requires: C, 44·4; H, 6·2 %.)

Preparation of an acetate. The polysaccharide (1 g.) was dissolved in water (1 ml.) and pyridine (25 ml.) added slowly. A small amount of inorganic material separated and was removed by centrifuging. Acetic anhydride (25 ml.) was now added slowly and the solution maintained at 40° during the addition (10 minutes). It was then cooled to 20° and left overnight. Next morning the clear pale yellow solution was poured into 200 ml. of warm water, and the acetate was obtained as a flocculent precipitate. It was centrifuged and washed repeatedly with warm water until free from the acetylation mixture. It was dried *in vacuo*. Yield, 1·6 g. Ash content, 0·9 %. OAc, 46·1 %. $[\alpha]_{5790} = +52{\cdot}6°$ in acetone

($c=1{\cdot}56$). Fractional precipitation of the acetate from acetone by means of ether showed that it was homogeneous.

Regeneration of the polysaccharide. The acetate (1 g.) was dissolved in acetone and a slight excess of N NaOH added. The product was precipitated twice from weak HCl solution, washed with absolute alcohol and dried *in vacuo*. Ash content, 0·7 %. $[\alpha]_{5790}=+62°$ ($c=1{\cdot}0$).

Properties of the purified polygalactose. This was a white hygroscopic powder containing 1·83 % of ash and 10 % of moisture; $[\alpha]_{5790}=-84{\cdot}3°$. It was analysed in the same manner as the polymannose and again the ash content prevented a satisfactory analysis.

(Found: C, 44·4; H, 7·0 %. $(C_6H_{10}O_5)_n$ requires: C, 44·4; H, 6·2 %.)

The solution in water was colourless, faintly acid to litmus and with no action on Fehling's solution or on iodine.

It readily hydrolysed as follows with 0·3N sulphuric acid. ($c=0{\cdot}711$.) 0 minutes $[\alpha]_{5790}=-84{\cdot}3°$, 10 minutes $[\alpha]_{5790}=-50{\cdot}0°$, 15 minutes $[\alpha]_{5790}=0°$, 20 minutes $[\alpha]_{5790}=+30{\cdot}0°$, 45 minutes $[\alpha]_{5790}=+72{\cdot}0°$, 60 minutes $[\alpha]_{5790}=+88{\cdot}0°$ (equilibrium value). Equilibrium value corrected for ash and as hexose $=+80{\cdot}8°$; $[\alpha]^{20}_{5790}$ for *d*-galactose $=+83{\cdot}6°$.

Acetylation. The polysaccharide (1 g.) was acetylated and the product isolated in the manner described for the polymannose. Yield, 1·5 g. of a white, ash-free powder. It had $[\alpha]_{5790}$ in acetone $=-72{\cdot}8°$ ($c=0{\cdot}75$). Acetyl content, 44·4 %. (Theoretical for the triacetate of a polyhexose, $C_6H_7O_2(OCOCH_3)_3$, 44·8 %.)

Regeneration of the polysaccharide by deacetylation with sodium hydroxide was unsatisfactory, the product being extremely hygroscopic.

II. *Investigation of the polysaccharides from Czapek-Dox medium.* The products were obtained by precipitation by acetone from the concentrated metabolism solution and isolated as previously described for Raulin-Thom medium. The ash content was very high (approximately 30 %), and the product was more soluble in water giving a clear yellow solution. The yields and properties were as follows.

Table VI.

Exp. No.	Yield in g.	% ash content	$[\alpha]_{5790}$ in water
5+6	102·3	30·0	0
7	51·6	31·2	−8·0°
10	45·6	28·5	−5·0°

Fractional precipitation from aqueous solution with alcohol removed most of the ash in the first fraction, and a series of fractions with different rotations was obtained. The crude material behaved on hydrolysis in a manner exactly similar to the product described from Raulin-Thom medium.

Hydrolysis of the crude polysaccharide with 0·5N H_2SO_4. A portion of the crude polysaccharide containing 9·5 % ash, and having an initial rotation of $[\alpha]_{5790}=-15{\cdot}3°$ dissolved ($c=0{\cdot}96$) in 0·5N H_2SO_4, and heated at 100° reached an equilibrium value of $[\alpha]_{5790}=+48°$ in 70 minutes.

Strong sulphuric acid was now added to make a 3N solution and the solution heated again at 100°. The rotation fell to an equilibrium value of $[\alpha]_{5790}=+35{\cdot}3°$ in 60 minutes.

The crude polysaccharide (3 g.) was hydrolysed with 0·3N H_2SO_4 as previously described. 2 g. of unhydrolysed polysaccharide were obtained on pouring the solution into absolute alcohol. This polysaccharide gave *d*-mannose on hydrolysis and after purification was identical with the polymannose previously described.

The mother-liquors on concentration and extraction with alcohol gave crystalline *d*-galactose, and it is probable that the crude polysaccharide is a mixture of the same polymannose and polygalactose as from the Raulin-Thom medium though this mixture has a higher polymannose content.

C. *Fractions III, IV and V (Ether extracts).*

Each of the above fractions, representing the material extracted by ether in the various experiments on both media, was allowed to stand for several weeks in air to ensure as complete crystallisation as possible. They were then separately ground in a glass mortar with a small amount of chloroform (25 ml.), the resulting dark brown suspension filtered and the insoluble portion washed with chloroform. In this way, each extract was separated into a quantity of white or light brown crystalline material and a chloroform mother-liquor which, after removal of chloroform, was dried down to a reddish brown syrup.

Using Czapek-Dox medium, the crystalline material obtained from fractions III and IV consisted of almost pure carlosic acid and from fraction V of almost pure carolinic acid, the total yields of which are given in Table VII. It appears therefore, that carlosic acid is completely extracted in the first three of the ten ether extracts, together with small amounts of carolinic acid, whilst the latter acid is extracted more slowly and is accumulated in the remaining ether extracts. This behaviour may be readily accounted for by considering the relative solubilities of these two acids in water and ether as shown later.

Using Raulin-Thom medium the crystalline material consisted in each of the fractions III, IV and V of a mixture of carolinic acid with varying but smaller amounts of verticillic acid, the total yields of which are given in Table VII. These two acids were then separated by fractionation with acetone in which verticillic acid is very much less soluble than carolinic acid.

Examination of the syrups obtained by drying down the chloroform mother-liquors from the above acids showed that in all cases appreciable amounts of carolic acid are present. In order to separate this acid, the combined syrups from experiments 5, 6, 7 and 10 on Czapek-Dox medium and, separately, the combined syrups from experiments 8, 9, 11 and 12 on Raulin-Thom medium were thoroughly triturated with water. The combined syrups from the Czapek-Dox medium were extracted with a total volume of 450 ml. of water and the syrups from the Raulin-Thom medium with 300 ml. of water.

The aqueous extracts were then filtered and extracted once with an equal volume of chloroform. The chloroform extracts were evaporated and the resultant yellowish oil seeded with carolic acid and set aside to crystallise. The crystalline residue was finally rubbed up with a little absolute ethyl alcohol, filtered and washed with a small amount of alcohol, when almost pure carolic acid resulted. By this treatment the combined syrups from the experiments 5, 6, 7 and 10 on Czapek-Dox medium (total weight after drying at 50° *in vacuo* = 250 g.) yielded 53·5 g. of yellowish oil from which 30 g. of carolic acid were obtained, while the combined syrups from experiments 8, 9, 11 and 12 on Raulin-Thom medium (total weight after drying at 50° *in vacuo* = 200 g.) yielded 35·4 g. of yellowish oil from which 18 g. of carolic acid were obtained.

Having removed the carolic acid from the combined syrups by water extraction as described above, the water-insoluble portion of the syrups was allowed to stand for four weeks when further crystallisation occurred, and small amounts of ramigenic acid separated in hard, well-formed prisms. These were filtered off, washed clean from syrup by rubbing up with chloroform and recrystallised

from acetone-light petroleum. The weights of pure ramigenic acid obtained from the syrups from the Raulin-Thom and the Czapek-Dox media were 3 g. and 1 g. respectively.

The syrups remaining after separation of ramigenic acid still contained small amounts of verticillic acid, amounting to about 7 % of the weight of the syrup from both media. In addition, in the case of the syrup from Czapek-Dox medium, but not from Raulin-Thom medium, a very small amount of carlosic acid was detected. These two acids were isolated by making use of specific precipitating reagents. Thus verticillic acid was separated by neutralising an aliquot portion of the syrup with sodium hydroxide to phenolphthalein and adding mercuric chloride which, as will be shown later, precipitates verticillic and ramigenic acids but does not precipitate the remaining four acids. The mercury precipitates from the two media were then suspended in water, the mercury removed with hydrogen sulphide, the excess of hydrogen sulphide removed in a stream of air and the solutions extracted with ether. After removal of the ether, the extracted material crystallised and was rubbed up with a small volume of acetone, filtered and washed with a little acetone. The residual crystalline material was shown by comparison with an authentic specimen to be verticillic acid. Finally, the presence of a very small amount of carlosic acid in the syrups from Czapek-Dox medium was detected by carefully adding normal lead acetate to the neutralised solution of an aliquot portion of the syrup. This reagent, as will be shown later, specifically precipitates carlosic acid, but its addition requires care as the precipitate redissolves in excess of the reagent. The lead precipitate was then rubbed up with $2N$ H_2SO_4 and the carlosic acid extracted with ether. After removal of the ether, the yellow crystalline mass was rubbed up with chloroform, filtered and washed with a little chloroform. The very small amount of crystalline material was shown by comparison with an authentic specimen to be carlosic acid $C_{10}H_{12}O_6$.

D. *Fractions VI and VII (Chloroform extracts).*

Each of the above fractions, representing the material extracted by chloroform in the various experiments on both media, was allowed to stand for several weeks in air to ensure as complete crystallisation as possible. They were then separately ground in a glass mortar with a small amount of absolute ethyl alcohol (25 ml.), the resulting dark brown suspension filtered and the insoluble portion washed with a little alcohol. In this way all the fractions VI gave a white crystalline powder consisting of almost pure carolic acid and amounting to 70 % of the total weight of the fractions, whilst all the fractions VII gave small yields of a white crystalline powder consisting of fairly pure carlic acid. The alcoholic mother-liquors from fractions VI and VII were evaporated *in vacuo* and extracted with water in which they were almost completely soluble. The aqueous solutions

Table VII. *Yields in g. of isolated and almost pure crystalline acids.*

Exp. No.	Medium	Carolic acid $C_9H_{10}O_4$	Carolinic acid $C_9H_{12}O_7$	Carlic acid $C_{10}H_{10}O_6$	Carlosic acid $C_{10}H_{12}O_6$	Verticillic acid $C_{26}H_{32}O_{12}$	Ramigenic acid $C_{16}H_{20}O_6$	
5	Czapek-Dox	44·7	8·2	7·5	9·3	6·7		
6	,,	43·7	10·2	5·4	9·0	4·4		
7	,,	44·6	3·9	1·7	5·6	5·9		1 g.
10	,,	42·4	7·8	5·3	9·6	3·3		
8	Raulin-Thom	29·7	16·3	4·1	—	13·6		
9	,,	27·9	14·9	3·0	—	13·2		
11	,,	29·6	14·5	1·3	—	11·5		3 g.
12	,,	26·7	16·6	1·1	—	4·2		

were then extracted once with an equal volume of chloroform, the chloroform removed and the partially crystalline residue washed with alcohol, further small amounts of carolic acid being thus obtained. The total yield of carolic acid obtained from fraction VI was thus raised to 75 % of the weight of this fraction.

The final yields of the practically pure crystalline acids in the eight typical experiments are given in Table VII.

Properties and analysis of the crystalline acids.

We propose now to give details of the general properties and analysis of the different crystalline acids isolated, reserving for future communications the question of their molecular constitution. The equivalents were determined by dissolving a weighed amount in a suitable solvent and titrating to phenolphthalein with *N*/10 sodium hydroxide. Optical rotations were determined at room temperature on the free acids in a suitable solvent and also on neutral aqueous solutions of the sodium salts using the mercury green line λ_{5461}. Solubilities were determined roughly by shaking 0·1 g. of the finely powdered material at room temperature with a gradually increasing and measured amount of the solvent in question until dissolution was just complete. The reactions of aqueous solutions of the sodium salts of the different acids towards a series of inorganic reagents were determined by adding to 2 ml. of a 1 % aqueous solution of the acid, neutralised to phenolphthalein with sodium hydroxide, 1 ml., drop by drop, of a 10 % aqueous solution of the reagent in question. All micro-analyses were carried out by Schoeller (Berlin).

Carolic acid $C_9H_{10}O_4$. Carolic acid is best purified by recrystallising (2 g.) from boiling ethyl alcohol (20 ml.). Yield 1·4 g. of colourless radiating needles, M.P. 132° without obvious decomposition. On resetting and remelting the M.P. gradually falls to a constant value of 113°.

(Found: C, 59·45, 59·42; H, 5·61, 5·58 %; OCH_3, nil; Mol. Wt. 217, 235. $C_9H_{10}O_4$ requires: C, 59·31; H, 5·54 %; Mol. Wt. 182.) The molecular weight found by the depression of the freezing-point of benzene was 196.

Titration. 0·2380 g. and 0·2318 g. required respectively 13·05 and 12·70 ml. *N*/10 NaOH, corresponding to equivalents of 182·4 and 182·5 respectively. ($C_9H_{10}O_4$ titrating as a monobasic acid requires 182·1.)

Rotations. (*a*) Free acid in water: ($c = 0·50$) $[\alpha]_{5461} = +84°$.

This value remained constant for 1 hour but had fallen after 24 hours to +81°; the solution becoming faintly yellow in colour.

(*b*) Sodium salt: ($c = 0·52$ calculated as monosodium salt) $[\alpha]_{5461} = +71·5°$.

This value did not change overnight and the solution did not become coloured.

Solubilities. 1 g. dissolved in 2 ml. chloroform, 12 ml. water or acetone, 28 ml. ethyl acetate, 36 ml. ethyl alcohol, 40 ml. benzene, 400 ml. ether, but was not completely dissolved in 500 ml. light petroleum, B.P. 40–50°.

Salts. Carolic acid gives no precipitate even on standing overnight with mercuric chloride, copper sulphate, uranium acetate, normal or basic lead acetate or with calcium or barium chloride even in presence of four volumes of alcohol. Silver nitrate gives no precipitate even in concentrated solution and no reduction on standing overnight. Ferric chloride gives a characteristic bright orange colour but no precipitate (*cf.* carolinic acid).

An aqueous solution of the acid is strongly acid to Congo red, reduces acid or neutral permanganate immediately in the cold and decolorises bromine water instantly. With Brady's reagent (2 : 4-dinitrophenylhydrazine in 2 *N* HCl), there is no immediate precipitate but on standing with excess of reagent a crystalline orange precipitate begins to separate after 48 hours and continues for 3 to 4 weeks. The acid dissolves in concentrated H_2SO_4 giving a colourless solution in the cold, remaining colourless at 100° but at 140–150° becoming first rose-pink, then darkening to a characteristic intense bluish red colour.

Carolinic acid $C_9H_{12}O_7$. ($C_9H_{10}O_6$, H_2O.) Carolinic acid is best purified by recrystallisation from acetone (or acetone-light petroleum) from which solvent it separates in colourless prisms, M.P. 123° with evolution of gas.

(Found: C, 46·53, 46·43; H, 5·26, 5·21 %; OCH_3, nil. $C_9H_{12}O_7$ requires: C, 46·53; H, 5·21%; Mol. Wt. 232.) The molecular weight of carolinic acid must be that corresponding to the formula $C_9H_{12}O_7$ since it readily loses $1H_2O$ giving a product having an observed molecular weight corresponding to $C_9H_{10}O_6$ (see below).

Titration. 0·1723 g. required 14·50 ml. *N*/10 NaOH corresponding to an equivalent of 117·($C_9H_{12}O_7$ titrating as a dibasic acid requires 116.)

Rotation. (*a*) Free acid in water: ($c = 0·34$) $[\alpha]_{5461} = +60°$. The rotation did not change overnight but the solution became faintly yellow in colour.

(*b*) Disodium salt: ($c = 0·41$; calculated as the disodium salt) $[\alpha]_{5461} = +54°$. The rotation did not change overnight and the solution remained colourless.

Solubilities. 1 g. dissolves in 25 ml. ethyl alcohol, 30 ml. acetone, 150 ml. water, 170 ml. ethyl acetate, but was not completely dissolved in 500 ml. benzene, chloroform, ethyl ether or light petroleum, B.P. 40–50°.

Salts. Carolinic acid gives no precipitate even on standing overnight with silver nitrate (and no reduction), mercuric chloride, uranium acetate or normal lead acetate. Ferric chloride gives an immediate brown precipitate, soluble in excess to a bright orange-coloured solution. Copper sulphate gives no immediate precipitate but on standing there is a copious separation of beautiful pure blue needles. Basic lead acetate gives a very heavy immediate precipitate completely soluble in excess. Barium or calcium chloride in presence of four volumes of ethyl alcohol gives a gelatinous precipitate tending to become crystalline on standing.

An aqueous solution of the acid is strongly acid to Congo red and only slowly reduces permanganate in the cold. With Brady's reagent it slowly gives a slight yellow precipitate. The acid dissolves in concentrated H_2SO_4 giving a colourless solution in the cold which becomes dark brownish red on heating to 140–150°.

Carolinic acid, crystallised from acetone, readily loses a molecule of water on drying at 80°. 0·2216 g. dried to constant weight at 80° lost 0·0170 g. $H_2O = 7·67$ % H_2O. (Theoretical for $C_9H_{12}O_7 \rightarrow C_9H_{10}O_6 = 7·76$ %.)

(Found for the dried substance: C, 50·81; H, 4·73 %; Mol. Wt. 223, 231; equivalent, 105. $C_9H_{10}O_6$ requires: C, 50·45; H, 4·71 %; Mol. Wt. 214; equivalent titrating as a dibasic acid, 107.) The dehydrated material on recrystallisation from acetone reverts to carolinic acid $C_9H_{12}O_7$.

Carlic acid $C_{10}H_{10}O_6$. Carlic acid is best purified by recrystallising from ethyl alcohol, from which it separates in colourless needles, M.P. 176°, giving a brown gummy melt which does not resolidify on cooling.

(Found: C, 53·23, 53·22; H, 4·53, 4·47 %; OCH_3, nil. $C_{10}H_{10}O_6$ requires: C, 53·08; H, 4·46 %; Mol. Wt. 226.) The molecular weight found by the depression of the freezing-point of dioxan was 240. The empirical formula of carlic acid $C_{10}H_{10}O_6$ also follows from that of carolic acid $C_9H_{10}O_4$ since, from observations which will be published later, it is known that the molecule of carlic acid contains CO_2 more than that of carolic acid.

Titration. 0·1214 g. required 10·70 ml. *N*/10 NaOH corresponding to an equivalent of 113·4. ($C_{10}H_{10}O_6$ titrating as a dibasic acid requires 113.)

Rotation. (*a*) Free acid in water: ($c = 0·28$) $[\alpha]_{5461} = -160°$. This value was not changed overnight and the solution remained colourless.

(*b*) Disodium salt: ($c = 0·29$; calculated as the disodium salt) $[\alpha]_{5461} = -147°$. This value was not changed overnight and the solution remained colourless.

Solubilities. 1 g. dissolved in 14 ml. water, 45 ml. acetone, 75 ml. ethyl alcohol, 270 ml. ethyl acetate, 350 ml. chloroform, 500 ml. benzene, but was not completely dissolved in 500 ml. ethyl ether, light petroleum, B.P. 40–50°, or butyl alcohol.

Salts. Carlic acid gives no precipitate even on standing overnight with silver nitrate (and no reduction), mercuric chloride, uranium acetate or normal lead acetate. Ferric chloride gives an immediate brownish-yellow precipitate soluble in excess to an orange-yellow solution. Copper

sulphate gives no immediate precipitate but after several days' standing blue-green nodular crystals separate, which are quite different in colour from those given by carolinic acid. Basic lead acetate gives a very heavy precipitate which is readily soluble in excess. Calcium chloride in presence of four volumes of alcohol does not give an immediate precipitate, but on standing for several days a moderate precipitate is formed. Barium chloride in presence of four volumes of alcohol gives an immediate heavy flocculent precipitate.

An aqueous solution is acid to Congo red, reduces acid permanganate fairly slowly and does not give a precipitate with Brady's reagent. The acid dissolves in concentrated H_2SO_4 giving a colourless solution in the cold, which becomes only pale brown on heating for some time at 140–150°.

Carlosic acid $C_{10}H_{12}O_6$. Carlosic acid is best purified by crystallisation from a large volume of boiling benzene from which it separates in fine colourless needles, M.P. 181°.

(Found: C, 52·51, 52·62; H, 5·35, 5·30 %; OCH_3, nil; Mol. Wt. 236, 242. $C_{10}H_{12}O_6$ requires: C, 52·61; H, 5·31 %; Mol. Wt. 228.)

Titration. 0·1040 g. required 9·20 ml. *N*/10 NaOH corresponding to an equivalent of 113·1. ($C_{10}H_{12}O_6$ titrating as a dibasic acid requires 114.)

Rotations. (*a*) Free acid in water: ($c=0·21$) $[\alpha]_{5461}=-160°$. This value was unchanged overnight and the solution did not become coloured.

(*b*) Disodium salt: ($c=0·25$ calculated as the disodium salt) $[\alpha]_{5461}=-150°$. This value was unchanged overnight and the solution did not become coloured.

Solubilities. 1 g. dissolved in 14 ml. acetone, 30 ml. ethyl alcohol, 45 ml. ethyl acetate, 90 ml. water, 250 ml. of chloroform or ether, 500 ml. benzene, but was not completely dissolved in 500 ml. light petroleum, B.P. 40–50°.

Salts. Carlosic acid gives no precipitate even on standing overnight with silver nitrate (and no reduction), mercuric chloride, copper sulphate or calcium chloride in presence of four volumes of ethyl alcohol. Ferric chloride gives an immediate brownish-yellow precipitate insoluble in excess. Uranium acetate gives no immediate precipitate but on standing for 2–3 days a characteristic copious semi-crystalline yellow precipitate separates. Both normal and basic lead acetates give immediate very heavy precipitates soluble in large excess. Barium chloride in presence of four volumes of alcohol gives a flocculent white precipitate.

An aqueous solution is acid to Congo red, reduces acid $KMnO_4$ fairly slowly, and gives an immediate orange-yellow crystalline precipitate with Brady's reagent, M.P. 135°. The acid dissolves in concentrated H_2SO_4 giving a colourless solution in the cold which becomes only pale brown on heating at 140–150°.

Ramigenic acid $C_{16}H_{20}O_6$. Ramigenic acid is precipitated on acidifying aqueous solutions of the sodium salt in large prisms containing $1H_2O$ (*a*), which is lost on standing *in vacuo* over P_2O_5 for several days. It crystallises in well-formed prisms from acetone-light petroleum (*b*). The acid $C_{16}H_{20}O_6,H_2O$ softens slightly at 125° owing to loss of water, but does not melt. The anhydrous acid melts at 171°.

(Found: (*a*) C, 59·24, 59·10; H, 6·62, 6·75 %; OCH_3, nil. $C_{16}H_{20}O_6$, H_2O requires: C, 58·86; H, 6·80 %. (*b*) C, 62·40, 62·30; H, 6·59, 6·51 %; OCH_3, nil. $C_{16}H_{20}O_6$ requires: C, 62·30; H, 6·54 %.)

Titration. 0·2343 g. of the acid $C_{16}H_{20}O_6$ was dissolved in 20 ml. absolute alcohol and titrated to phenolphthalein. The first pink flush (rapidly disappearing) was obtained after adding 7·70 ml. *N*/10 NaOH corresponding to an equivalent of 304 ($C_{16}H_{20}O_6$ titrating as a monobasic acid requires 308). A further 12·30 ml. *N*/10 NaOH (total 20·0 ml.) were added and the solution heated on the water-bath for 2 hours. On cooling and titrating, the red colour first disappeared after adding 5·24 ml. *N*/10 H_2SO_4, corresponding to an equivalent of 158·8 ($C_{16}H_{20}O_6$ titrating as a dibasic acid requires 154). On standing, the solution became slowly pink, was decolorised by addition of a few drops of *N*/10 H_2SO_4 and, on standing, again became pink. The process was repeatable, indicating that the more difficultly opened acidic group responsible for the dibasic character closes with great ease in more acid solution. The solution was then acidified and allowed to stand. Well-formed prisms having the characteristics of ramigenic acid separated, M.P. 171° (after loss of water at 125°) and not depressed on mixing with an authentic sample.

Rotations. (*a*) Free acid in alcohol: ($c=0{\cdot}22$) $[\alpha]_{5461}=+28°$. The rotation did not change overnight, and the solution did not become coloured.

(*b*) Sodium salt: ($c=0{\cdot}27$; calculated as monosodium salt) $[\alpha]_{5461}=+85°$. The rotation did not change overnight, and the solution remained colourless.

Solubilities. 1 g. dissolved in 14 ml. acetone, 16 ml. ethyl alcohol, 30 ml. chloroform, 90 ml. ethyl acetate, 180 ml. ether, but was not completely dissolved in 500 ml. water, benzene or light petroleum, B.P. 40–50°.

Salts. Ramigenic acid gives no precipitate with copper sulphate, uranium acetate or with calcium or barium chloride even in presence of four volumes of alcohol. Silver nitrate is slowly reduced in the cold, giving a silver mirror on standing overnight. Ferric chloride gives a heavy pale brown crystalline precipitate, probably of ramigenic acid, the supernatant liquid being only light yellowish brown in colour. Mercuric chloride gives an immediate yellow colour with slow development of a heavy pale yellow precipitate. Normal and basic lead acetates give slight white precipitates.

The acid is insoluble in water and an aqueous suspension is not acid to Congo red and reduces $KMnO_4$ immediately in neutral and more slowly in acid suspension. No reaction occurs with Brady's reagent, but this may be due to the insolubility of the acid. The acid dissolves in concentrated H_2SO_4 giving a colourless solution in the cold which becomes pale yellow at 100° and dark brown at 140–150°.

Verticillic acid $C_{26}H_{32}O_{12}$. Verticillic acid is best purified by allowing the boiling saturated acetone solution to stand overnight, when the acid separates in fine needles, M.P. 171°, giving a brown viscous melt which does not reset on cooling.

(Found: C, 58·06, 58·24; H, 6·04, 6·06 %; OCH_3, nil. $C_{26}H_{32}O_{12}$ requires: C, 58·17; H, 6·02 %.)

Titration. Verticillic acid on titrating in alcoholic solution behaves like an acid lactone; 0·1578 g. dissolved in 20 ml. alcohol gave the first pink flush after the addition of 11·09 ml. *N*/10 NaOH. The titration then became slow as for a lactone. After standing for three hours at room temperature with an excess of NaOH, the final titration was 11·74 ml. *N*/10 NaOH, corresponding to an equivalent of 134·5 ($C_{26}H_{32}O_{12}$ titrating as the lactone of a tetrabasic acid requires 134).

Rotations. (*a*) Free acid in alcohol: ($c=0{\cdot}40$) $[\alpha]_{5461}=-53°$. This value rapidly fell on standing and the solution became progressively more and more yellow in colour. The specific rotations after 10, 15 and 45 minutes, 2, 5, 24, 48 and 72 hours were respectively −47°, −44°, −36°, −35°, −34°, −28°, −20°, −14°.

(*b*) Sodium salt: ($c=0{\cdot}49$, calculated as the tetrasodium salt) $[\alpha]_{5461}$ initially $=+46°$ and only slowly decreases to +41° after 24 hours, the solution becoming faintly yellow in colour.

Solubilities. 1 g. dissolved in 45 ml. ethyl alcohol, 270 ml. acetone, but was not completely dissolved in 500 ml. water, benzene, chloroform, ethyl acetate, ether and light petroleum, B.P. 40–50°.

Salts. Silver nitrate gives no precipitate, but reduction commences after 2–3 hours in the cold with the formation of a silver mirror overnight. Mercuric chloride gives an immediate heavy white precipitate with a yellow supernatant fluid. Ferric chloride gives a characteristic very intense brownish-red colour. Uranium acetate gives no precipitate but a characteristic orange-yellow colour. Slight precipitates are given with copper sulphate, normal and basic lead acetates and calcium or barium chloride in presence of four volumes of alcohol.

An aqueous solution of verticillic acid is faintly acid to Congo red and reduces acid permanganate very readily in the cold. An alcoholic solution of the acid gives no immediate precipitate with Brady's reagent, but an orange precipitate begins to separate after 12 hours. The acid dissolves in concentrated H_2SO_4 giving a colourless solution in the cold which becomes pale yellow at 100° and dark brown at 140–150°.

The molecular weights by the Rast camphor method for both verticillic and ramigenic acids were unsatisfactory, owing to the fact that they both decompose on heating at a temperature below that of the melting-point of camphor. The molecular formulae $C_{26}H_{32}O_{12}$ and $C_{16}H_{20}O_6$ have been assigned to verticillic and ramigenic acids respectively for the following reasons:

(*a*) Verticillic acid (1 molecule) on mild hydrolysis gives rise to 1 molecule of ramigenic acid and a keto-acid which analyses for the empirical formula of $(C_5H_6O_3)_n$.

(*b*) Ramigenic acid titrates in the cold as a monobasic acid, equivalent 304 ($C_{16}H_{20}O_6$ requires 308).

(*c*) Ramigenic acid with diazomethane gives a crystalline monomethyl derivative containing 9·60 % OCH_3 and having an observed molecular weight by the Rast camphor method of 332 and 340 ($C_{16}H_{19}O_5OCH_3$ requires 9·63 % OCH_3 and molecular weight 322). It follows therefore that the empirical formula for ramigenic acid must be $C_{16}H_{20}O_6$ and hence that for verticillic acid is $C_{26}H_{32}O_{12}$.

Summary and discussion.

The metabolic products of a new mould species isolated from mouldy Italian maize, *Penicillium Charlesii* G. Smith, have been investigated. This mould was grown at 24° on Czapek-Dox medium in which glucose is the only source of carbon, and on Raulin-Thom medium in which glucose is the only carbohydrate present. The metabolic products isolated consist of two new polysaccharides and six hitherto undescribed optically active organic acids.

The polysaccharides are a polygalactose $[\alpha]_{5790} = -84°$ and a polymannose $[\alpha]_{5790} = +63°$ which give on hydrolysis *d*-galactose and *d*-mannose respectively as the only carbohydrates. Both of these polysaccharides were formed on both media and their production in considerable amounts affords an interesting example of the ready conversion by a mould of glucose into mannose and galactose.

The six organic acids isolated are as follows: (*a*) carolic acid $C_9H_{10}O_4$, (*b*) carolinic acid $C_9H_{12}O_7$, (*c*) carlic acid $C_{10}H_{10}O_6$, (*d*) carlosic acid $C_{10}H_{12}O_6$, (*e*) ramigenic acid $C_{16}H_{20}O_6$ and (*f*) verticillic acid $C_{26}H_{32}O_{12}$. Of these (*a*), (*b*) and (*e*) are dextrorotatory while (*c*), (*d*) and (*f*) are laevorotatory. The acids were isolated from the metabolism solution and separated from each other in a state of purity by taking advantage of their differing solubilities and of their varying precipitability by different metallic salts.

It is a point of considerable microbiological interest to compare the products of metabolism of three species of *Penicillium*, all of which were isolated from mouldy maize, *i.e.* of *P. brevi-compactum* Dierckx (syn. *P. stoloniferum* Thom), *P. Charlesii* G. Smith, each of which was isolated from mouldy Italian maize, and *P. puberulum* Bainier from mouldy American maize. *P. brevi-compactum* produces from glucose the following series of optically inactive phenolic acids, each containing a benzenoid nucleus: $C_{17}H_{20}O_6$, $C_{10}H_{10}O_7$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_5$ and $C_8H_6O_6$ [Clutterbuck *et al.*, 1932; Oxford and Raistrick, 1932; 1933, 1, 2; Clutterbuck and Raistrick, 1933]. *P. Charlesii* on the other hand produces from glucose the following series of optically active non-benzenoid acids; $C_{26}H_{32}O_{12}$, $C_{16}H_{20}O_6$, $C_{10}H_{12}O_6$, $C_{10}H_{10}O_6$, $C_9H_{12}O_7$ and $C_9H_{10}O_4$. It will be noticed that two acids having the empirical formula $C_{10}H_{10}O_6$, but differing widely in chemical constitution, are produced by *P. brevi-compactum* and *P. Charlesii*, which curiously enough were isolated from the same sample of mouldy Italian maize. *P. puberulum* produces from glucose three organic acids, penicillic acid $C_8H_{10}O_4$ [Alsberg and Black, 1913], puberulic acid $C_8H_6O_6$, and an acid $C_8H_4O_6$ [Birkinshaw and Raistrick, 1932]. Each of these three acids is optically inactive and non-benzenoid in nature.

In view of the amazing variety of the metabolic products of different species of Penicillia occurring in mouldy maize, some of which are known to be definitely toxic, it is tempting to enquire whether the possibility has been entirely ruled out that mould infection of maize has a connection with human pellagra. Most workers on human pellagra appear to agree that this disease is intimately connected in some way with the consumption of maize. Aykroyd [1933] divides the views which have been expressed as to the origin of pellagra under three headings:

(*a*) theory of a maize toxin,

(*b*) theory of amino-acid deficiency due to the low biological value of maize proteins,

(*c*) theory of vitamin deficiency.

It would be outside the scope of this paper to discuss (*b*) and (*c*), but we venture to raise the question as to whether the presence of toxic materials elaborated by organisms infecting maize may not form a contributory factor. In this connection it is of interest to note, to quote Aykroyd (page 343), that "Italian antipellagra policy is enshrined as a law passed in 1902 which was based on the theory of causation generally accepted at that date. The sale of 'spoiled' (*i.e.* infected) maize as human food was strictly prohibited, and local authorities were forced to acquire public desiccators and municipal reservoirs in which maize could be kept in good condition." This is particularly interesting in view of the fact that the death rate per 100,000 of population from pellagra reported officially in Italy was 10·7 in the period 1896–1900, a figure which steadily decreased to 0·2 in 1928. On the other hand, in the United States of America where, so far as we are aware, no such law is in force as exists in Italy, the death rate per 100,000 in 1924 was 2·5, increasing steadily to 6·4 in 1928 and being 5·9 in 1930.

We desire to express our sincere thanks to our colleague Dr A. E. Oxford for the determination of the molecular weights of carolic and carlic acids, and to Mr W. K. Anslow for much technical assistance during the course of this work.

REFERENCES.

Aykroyd (1933). *Nutrition Abstr. Rev.* **3**, 337.
Alsberg and Black (1913). *U.S. Dept. Agric., Bureau of Plant Industry, Bulletin No.* **270**.
Birkinshaw and Raistrick (1932). *Biochem. J.* **26**, 441.
Clutterbuck, Oxford, Raistrick and Smith (1932). *Biochem. J.* **26**, 1444.
—— and Raistrick (1933). *Biochem. J.* **27**, 654.
Koppeschaar (1876). *Z. anal. Chem.* **15**, 233.
Oxford and Raistrick (1932). *Biochem. J.* **26**, 1902.
—— —— (1933, 1). *Biochem. J.* **27**, 634.
—— —— (1933, 2). *Biochem. J.* **27**, 1473.
Raistrick and Smith (1933). *Biochem. J.* **27**, 96.
Smith (1933). *Trans. Brit. Mycol. Soc.* **18**, 90.
Thom (1930). The Penicillia. (London, Baillière, Tindall and Cox.)

XVII. THE VITAMIN D ACTIVITY OF BUTTER. II. THE COMPLEX NATURE OF THE ANTIRACHITIC ACTION OF BUTTER.

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In the first paper of this series [Kon and Booth, 1933, 2] evidence was brought forward to show that the antirachitic activity of autumn and winter butter cannot be quantitatively recovered in the non-saponifiable residue of these butters. Under exactly similar experimental conditions irradiated ergosterol or cod-liver oil could be subjected to saponification either alone or mixed with winter butter without loss of potency, *i.e.* their non-saponifiable residues carried the whole of their antirachitic activities.

Since then we have studied the effect of saponification on butters produced under summer conditions and also on butters rendered highly antirachitic either by direct irradiation with the quartz mercury vapour lamp, or by the feeding to cows of large amounts of irradiated yeast or of cod-liver oil. We have also attempted to concentrate the antirachitic activity of these various butters and of cod-liver oil by the method of alcoholic extraction used by Zucker [1922–23; 1925; 1928] for the preparation of an active concentrate from cod-liver oil.

Experimental.

Methods.

Most of the methods used for the saponification of butter for the preparation of olive oil dilutions of the non-saponifiable residues and for the animal tests have been described in the previous paper [Kon and Booth, 1933, 2]. The short saponification method was used throughout. For feeding, the non-saponifiable residues, Zucker extracts and other substances of which the doses given were too small for administration by 1 ml. calibrated volumetric pipettes were dissolved in ether and mixed with a small quantity of olive oil. After evaporation of the ether *in vacuo*, the remainder of the requisite amount of olive oil was added, the solution was mixed by the aid of a vigorous stream of nitrogen and fed by means of standardised pipettes delivering drops weighing from 19 to 21 mg. The dilution was so adjusted that the requisite dose of non-saponifiable residue or other material was present in from 1 to 4 drops of the solution.

In the distribution of animals for the feeding tests an endeavour was made to divide the litters as evenly as possible among the various substances undergoing test, and as a rule litter-mates were placed in all groups where comparison was especially desirable, such as the comparison between one level of raw butter and its equivalent as non-saponifiable residue. In these, as well as in the previous experiments [Kon and Booth, 1933, 2], not only was the degree of protection

afforded to rats by the various substances measured by the amount of ash in the dry defatted femora and humeri, but photographs were also taken, by the technique described in our previous paper, of sections of radii and ulnae stained with silver nitrate. The large number of rats used in the present tests precludes the publication of these photographs. The results arrived at by a study of these photographs were, in general, in excellent agreement with those based on the ash percentage.

Experiments carried out in the summer of 1933.

Methods. (*a*) *Summer butter from cows at pasture.* The cows went out to grass on April 20th, 1933. Butters from seven bi-weekly churnings were collected between May 12th and June 5th and aliquot parts of these butters, after rendering and filtering, were pooled. This period was very sunny throughout, there being an average of 5·6 hours of sunshine daily, to all of which the cows were exposed. The butter was saponified in the usual way on 3 occasions during the course of the experiment, the non-saponifiable residue being extracted with 4 portions of ether instead of the usual 3.

(*b*) *Butters from cows receiving irradiated yeast*[1]. Two groups of 2 Shorthorn cows each were maintained throughout the winter stall-feeding period 1933 on a ration identical with that fed to the typical herd of Shorthorns [Golding *et al.*, 1932]. Towards the end of this period the ration of one group was supplemented with 50 g. of irradiated yeast daily, while the supplement to the other group was 500 g. daily. In the case of group I, after 14 days of such supplementary feeding butter was made from the cream of the mixed milk of the 2 cows over a 2-day period. The butter was made in the case of group II from a similar sample of cream from the mixed milk of the 2 cows after the yeast had been fed for a period of 3 weeks. These butters were rendered and filtered in the usual way and kept in cold storage until required. They are hereinafter referred to as low-level and high-level yeast butter respectively.

(*c*) *Butter from cows receiving cod-liver oil*[1]. The butter was produced at the same time and under the same conditions as that of the cows receiving irradiated yeast. This group of 2 cows was given, however, daily supplements of 4 to 6 oz. of cod-liver oil for one week and 8 oz. daily per cow for 2 following weeks before the butter was collected.

(*d*) *Irradiated butter.* In preparing this butter we endeavoured to duplicate as far as possible the method of Steenbock and Wirick [1930] with the exception that we used for irradiation a sample of winter butter instead of June butter as used by these authors. The sample of winter butter collected between March 31st and April 4th, 1933, was rendered and filtered in the usual way and stored until required. After melting, 45·36 ml. of the fat were run into a circular bright nickel-plated tray 19 cm. in diameter, thus giving a layer of fat 0·16 cm. thick. The butter was maintained in the fluid state and at a temperature not exceeding 40° by being placed over a hot water-bath during irradiation. This was carried out for ½ hour with a K.B.B. atmospheric type quartz mercury vapour lamp situated at a distance of 45·7 cm. from the melted fat. The lamp had previously been in use for about 200 hours and consumed 2·5 amp. with a potential across the burner of 132 V. After irradiation the butter was kept in cold storage and, when necessary, saponified in the usual way.

(*e*) *Alcoholic extract of winter butter prepared by a method similar to that of Zucker* [1922–23; 1925; 1928]. 250 g. of pooled butter from bi-weekly samples

[1] The experiments dealing with the feeding to cows of irradiated yeast and of cod-liver oil will be reported in detail elsewhere.

collected between March 13th and 27th, 1933, were heated with 150 ml. of absolute ethyl alcohol until completely dissolved. The solution was vigorously shaken and allowed to cool and separate, and the alcoholic layer was then poured off. This extraction was followed by another with 100 ml. of 96 % alcohol and by two further extractions each with 75 ml. of 96 % alcohol. The combined alcoholic extracts were evaporated on the water-bath yielding 17·7 g. of a yellow oil. A part of this oil was saponified in the usual way. The residue from the alcoholic extraction was heated to about 45° and washed in a separating funnel with three successive portions of 100 ml. of hot water. It was then filtered and stored.

In Table I are given the results of the tests.

Table I. *Summer 1933. Prophylactic experiment. Antirachitic effect of various butters, of non-saponifiable residues from these butters and of an alcoholic extract and residue from one of them. Percentage of ash in dry defatted femora and humeri.*

The figures for ash percentage are averages of duplicate determinations on groups of 4 rats.

In the Tables the dosages of non-saponifiable residues, Zucker extracts, *etc.*, are expressed in the equivalent of the butter from which they were derived.

Positive controls	52·0 % ash
Negative controls (7 rats)	31·2 % ash

Substance tested	Dosage g.	Ash %
Summer butter	0·1	36·1
,,	0·2	42·8
,,	0·5	49·5
,,	1·0	53·5
Non-saponifiable residue of summer butter, equiv.	0·1	36·1
,, ,, ,,	0·2	38·7
,, ,, ,,	0·5	44·0
,, ,, ,,	1·0	49·8
High-level yeast butter	0·01	46·0
,,	0·03	50·4
,,	0·10	51·1
,,	0·25	52·9
Non-saponifiable residue of high-level yeast butter, equiv.	0·01	45·2
,, ,, ,,	0·03	50·3
,, ,, ,,	0·10	50·8
,, ,, ,,	0·25	52·4
Low-level yeast butter	0·05	38·0
,,	0·15	49·7
,,	0·50	53·5
Non-saponifiable residue of low-level yeast butter, equiv.	0·05	39·1
,, ,, ,,	0·15	46·0
,, ,, ,,	0·50	50·6
Cod-liver oil butter	0·10	38·1
,,	0·30	48·6
,,	1·00	51·8
Non-saponifiable residue of cod-liver oil butter, equiv.	0·10	38·0
,, ,, ,,	0·30	42·5
,, ,, ,,	1·00	48·3
Irradiated butter	0·018	49·8
,, ,, ,,	0·036–0·072	50·4
Non-saponifiable residue of irradiated butter, equiv.	0·018	49·9
,, ,, ,,	0·036–0·072	50·4
Winter butter	1·00	48·0
Zucker extract of winter butter, equiv.	1·00	35·2
Non-saponifiable residue of Zucker extract of winter butter, equiv.	1·00	35·1
Residue of winter butter after Zucker extraction, equiv.	1·00	46·9

It is apparent from the table that neither the high-level yeast butter nor the irradiated butter has lost its potency as a result of saponification. In the case of the irradiated butter the experiment is unfortunately rather unsatisfactory. Owing to a misjudgment of the degree of protection afforded to the rats receiving the irradiated butter it was decided on the 15th day of the experiment to double the dosage in the case of the animals receiving the higher level of butter and obviously also in the case of the animals receiving the corresponding level of non-saponifiable residue. When the rats were killed it was found that the earlier diagnosis *in vivo* was quite erroneous and that not only the increased higher dosage but also the lower dosage had afforded a high degree of protection. It is therefore possible that, in the case of this butter, some destruction of the antirachitic factor might have taken place upon saponification, but that since the lower dosage was almost sufficient for complete protection, any slight destruction of antirachitic factor would not be apparent.

In the case of the low-level yeast butter, no destruction is evident at the lowest dosage. On the other hand both the 0·15 and 0·5 g. levels of this yeast butter yield a definitely higher ash percentage than their corresponding non-saponifiable residues.

A very similar condition prevails in the case of the cod-liver oil butter. There is no difference between butter and non-saponifiable residue at the 0·05 g. level, but here again, two higher dosages of butter yield higher ash percentages than their corresponding non-saponifiable residues.

The same statement holds true in the case of summer butter.

It will be noticed that the antirachitic activity of summer butter withstands saponification to a greater extent than that of autumn and winter butters. Our observations on butters collected in summer 1933 are in complete agreement with a preliminary test carried out on butter collected in the summer period 1932 previously recorded by us [Kon and Booth, 1933, 1].

With regard to the Zucker extract of winter butter it is quite evident that, as far as this experiment goes, it would confirm Zucker and Barnett's opinion [1922–23], quoted in our previous paper [Kon and Booth, 1933, 2], that the antirachitic activity of butter cannot be concentrated by their method. It is obvious that the loss is not due primarily to destruction of the antirachitic factor by saponification, as practically the whole of the activity was left in the residue, and the saponification did not in this case further diminish the already slight activity of the alcoholic extract.

Experiments carried out in autumn 1933, first series.

In this period we repeated our summer experiments with irradiated butter using dosages of 5 and 10 mg. These dosages were intended to afford an incomplete protection in order to obtain an unequivocal result as to whether or not destruction occurred in this butter on saponification. We also prepared and tested in this period Zucker extracts of high-level yeast butter, irradiated butter, winter butter and cod-liver oil. It was obvious that before reaching any definite conclusions with regard to the inapplicability of Zucker's method to butter, we had to make sure that we could obtain by this method, and under our laboratory conditions, an active concentrate from cod-liver oil. As an additional check on our previous findings as to the effect of saponification upon the antirachitic potency of various butters we again saponified a sample of winter butter.

Methods. (*a*) *Irradiated butter.* The same sample was used as in the previous summer experiment. This had been kept in the ice-chest throughout the intervening period. The methods of saponification and of feeding the butter and its

non-saponifiable residue were exactly as previously described, but four ethereal extractions of the non-saponifiable residue were made from the saponification mixture.

(*b*) *Preparation of the Zucker extracts.* For the preparation of Zucker extracts we used the same high-level yeast butter and irradiated butter as in our summer experiment. The cod-liver oil was from the bulk given us by Dr Coward for use in our previously reported experiments [Kon and Booth, 1933, 2], while the winter butter was from a pooled sample collected bi-weekly between February 10th and March 14th, 1933. The Zucker extracts of all these substances were made in exactly the same way. 20 g. of the substance to be extracted (200 g. in the case of winter butter) were mixed with 25 ml. of absolute alcohol (250 ml. in the case of winter butter) and were shaken on a mechanical shaker for 1½ hours. The butter-alcohol mixtures were, before shaking, heated sufficiently to melt the butter-fat. In all, four extractions were made, the same amount of alcohol being used for the second extraction as for the first, while in the last two extractions the amount of alcohol used was halved. After each of the first three extractions the oils were allowed to settle, and the alcoholic layer was siphoned off; after the last extraction the mixtures were centrifuged before the alcoholic layer was siphoned off. In each case the bulked extracts were concentrated on the water-bath, while the residues were washed four times with hot distilled water. The following yields were obtained:

Cod-liver oil	2 g. extract from 20 g. original oil.
Irradiated butter	5·08 g. extract from 20 g. original butter-fat.
High-level yeast butter	5·69 g. extract from 20 g. original butter-fat.
Winter butter	52·15 g. extract from 200 g. original butter-fat.

No special effort was made to recover the residues quite quantitatively, as the amounts used were far in excess of the requirements for the feeding tests, but for the purposes of dilution and feeding it was always assumed that the weight of the residue was equal to the weight of the original substance less the weight of the extract. It will be noticed that this method of extraction, which follows almost exactly that recommended by Zucker [1925; 1928], gives a much greater yield of extract from butter than the modification used in our summer experiment.

The methods of saponification and feeding of the Zucker extracts and residues and of their non-saponifiable residues were as previously described. The same applies to the saponification and feeding of winter butter; in all cases the soaps after saponification were extracted with four successive quantities of ether.

In Table II are given the results of these tests.

It can be seen that neither of the two levels of irradiated butter fed this time was sufficient to bring about complete protection. While the non-saponifiable residue of the lower level gives a slightly lower percentage of ash than the butter itself the reverse is true in the case of the higher level, and as these differences are well within the experimental error it is obvious that the antirachitic factor of irradiated butter can be quantitatively recovered in the non-saponifiable residue of the latter.

The saponification of winter butter has again resulted in a very incomplete recovery of its antirachitic activity in the non-saponifiable residue.

The results with the Zucker extracts have been rather unexpected. It is obvious from our figures that our inability to extract the vitamin D potency by this method is not restricted to winter butter alone, or even to butters of an enhanced antirachitic potency completely recoverable after saponification, but extends to cod-liver oil. It will be noticed that, while in the case of the latter the

Table II. *Autumn 1933. Prophylactic experiment, first series. Antirachitic effect of Zucker extracts and residues from cod-liver oil and various butters before and after saponification; antirachitic effect of the original butters and cod-liver oil and of the non-saponifiable residues of two of the butters. Percentage of ash in dry defatted femora and humeri.*

The figures for ash percentages are averages of duplicate determinations on groups of 4 rats.

Negative controls 34·9 % ash

Substance tested	Dosage g.	Ash %
High-level yeast butter	0·010	44·8
Zucker residue of high-level yeast butter, equiv.	0·010	33·7
Zucker extract of high-level yeast butter, equiv.	0·010	35·7
Non-saponifiable residue of Zucker extract of high-level yeast butter, equiv.	0·010	35·9
Cod-liver oil	0·0008	48·4
Zucker residue of cod-liver oil, equiv.	0·0008	44·7
Zucker extract of cod-liver oil, equiv.	0·0008	32·9
Non-saponifiable residue of Zucker extract of cod-liver oil, equiv. ...	0·0008	33·9
Irradiated butter	0·005	44·8
"	0·010	46·9
Non-saponifiable residue of irradiated butter, equiv.	0·005	43·7
" "	0·010	47·6
Zucker residue of irradiated butter, equiv.	0·010	35·9
Zucker extract of irradiated butter, equiv.	0·010	37·9
Non-saponifiable residue of Zucker extract of irradiated butter, equiv.	0·010	38·0
Winter butter	0·700	47·3
Non-saponifiable residue of winter butter, equiv.	0·700	35·4
Zucker residue of winter butter, equiv.	0·700	37·2
Non-saponifiable residue of Zucker residue of winter butter, equiv. ...	0·700	33·6
Zucker extract of winter butter. equiv.	0·700	36·4
Non-saponifiable residue of Zucker extract of winter butter, equiv. ...	0·700	33·1

residue after extraction seems to have retained a large part of its antirachitic potency, in the case of all the butters both the extract and the residue are only very slightly active. But even here the extract and residue of winter butter have suffered further loss on saponification.

Shipley *et al.* [1924] report that alcoholic extracts of butter-fat may contain appreciable amounts of antirachitic potency while the alcohol-insoluble residue was found by them to be inactive.

Experiments carried out in autumn 1933, second series.

Autumn butter collected in this period was tested for its antirachitic potency before and after saponification. The results of these tests were again in complete agreement with our previous experience with autumn and winter butters. At the same time, other experiments were carried out. These will be reported in the following paper.

Methods. The butter used in this experiment was collected between October 16th and November 16th, 1933. Five saponifications were carried out, the last four on the previous week's churnings, while in the first one a 3 days' sample was used. In order to ensure as perfect a recovery as possible the non-saponifiable residue was extracted on these occasions 5 times with ether.

The autumnal rains which followed the very dry and hot summer of 1933 had produced an exceptionally good crop of grass for this time of the year, and the butters, especially the first 2 or 3 samples, were therefore much more highly coloured than is usual under our conditions at this season. In fact the yellow

pigmentation and the vitamin A content of these butters were higher than those of the summer butter tested in the first series of experiments reported in this paper. On the other hand the actual sunshine to which the cows were exposed was in this case only 3·16 hours daily. The intensity of solar radiation at this time of the year was also obviously less than in summer. Observations on the colour and vitamin A activity of these butters will be reported in detail elsewhere. It might be mentioned here, incidentally, that the depth of yellow coloration and/or of vitamin A activity of a butter cannot be used as a reliable guide to its vitamin D activity.

In Table III are given the results of the tests.

Table III. *Autumn 1933. Prophylactic experiment, second series. Antirachitic effect of autumn butter and of its non-saponifiable residue. Percentage of ash in dry defatted femora and humeri.*

The figures for ash percentages are averages of duplicate determinations on groups of 4 rats.

Positive controls (2 drops of c.l.o. daily)	52·3 % ash
Negative controls	33·2 % ash

Substances tested	Dosage g.	Ash %
Autumn butter	0·25	40·8
,,	0·50	47·1
,,	1·00	52·9
Non-saponifiable residue of autumn butter, equiv.	0·25	34·6
,, ,,	0·50	39·1
,, ,,	1·00	40·3

This autumn butter is more potent than that collected at the same time during the previous year; on the other hand it is less potent than the sample of butter collected and tested in summer 1933. The loss of potency after saponification is quite marked and, as the non-saponifiable matter had been in this case so thoroughly extracted, this loss of potency in the course of saponification must be real and cannot be due to faulty extraction.

Experiments carried out in autumn 1933, third series.

The experiments carried out in this series were chiefly of a routine nature and formed part of our regular estimation of the antirachitic factor of butter by the curative line test technique. This latter has been described in our previous paper [Kon and Booth, 1933, 2]. The autumn butter was fed at three levels: 0·25, 0·5 and 1·0 g. daily before and after saponification.

The two lower levels of butter failed to give any definite signs of healing while 4 out of 5 rats receiving 1·0 g. of butter showed such signs varying in intensity from an indication of a line to a medium broken line. The lower potency of the non-saponifiable residue is shown by the fact that in only 2 out of 5 rats receiving the highest level are specks of calcification to be found.

Discussion.

Before devoting our attention to what we believe to be the positive results of our work, we would like to discuss briefly a negative finding, *i.e.* our inability to obtain an active extract from cod-liver oil by the method employed by Zucker [1922–23; 1925; 1928]. When in the summer of 1933 we prepared the Zucker extract

from butter and found it inactive we thought that this finding very definitely lent support to our contention that a chemical difference exists between the antirachitic agent of butter and other antirachitic agents. Obviously the validity of this finding stood or fell with our ability to obtain by the same method an active extract from cod-liver oil. Our failure to obtain such an extract is definitely not due to a destruction in the course of extraction, as the residue after extraction was found to contain the major part of the original activity. That highly active concentrates can be, and are, prepared from cod-liver oil by the method of Zucker we do not doubt. Such extracts are produced under Zucker's patents [Zucker, 1925; 1928] on a large scale in the United States and are, as Zucker [1933, 1, 2] reports, regularly used for enhancing the antirachitic potency of bread and milk.

We do not know what grades of cod-liver oil are used in this process. The cod-liver oil unsuccessfully used by us was one of very high vitamin D content (250–300 International units per g.) and was a high grade medicinal oil kindly given us by Dr Coward. It is conceivable that the acidity of the oil may play a vital part in the process of extraction, and that vitamin D could be extracted from oils of high acidity but not from neutral oils. This is merely an assumption, but the fact remains that our results in this respect are of no use in elucidating the difference between the antirachitic factor of butter and that from other sources.

In our first paper [Kon and Booth, 1933, 2] we reported observations on the effect of saponification on butters collected in autumn and winter. We observed that only a small fraction of the original antirachitic activity of these butters could be recovered in the non-saponifiable residues. At that time we abstained from making any suggestions regarding the possible abnormal behaviour of the antirachitic activity of butters in general, especially as we had been led by preliminary experiments [Kon and Booth, 1933, 1] to believe that butters may differ in this respect. Since then we have examined a variety of butters, and we have found that while the activity of some of them is markedly reduced by saponification, the antirachitic potency of others will withstand this treatment just as well as those of cod-liver oil and irradiated ergosterol.

When the butters examined by us are placed in an ascending series according to the degree of recovery in the non-saponifiable residue of their original antirachitic activity, it becomes at once evident that these butters are also graded in another respect—namely that they have been simultaneously arranged in ascending order of original antirachitic potency. In other words, the greater the potency of the butter, the more complete in our experience is the recovery of this potency after saponification.

The loss of potency on saponification is never complete, a loss of about 80 % of the original potency seems to be the maximum we have experienced. Such is the case with the least potent of the butters collected in autumn and winter. Any increase of potency above this practically basal level is accompanied by a corresponding, and, apparently, quantitive increase in the activity recoverable after saponification. We are therefore led to believe that at least two factors antirachitic for the rat are present in the butters which we have so far examined. One of them is clearly destroyed or diverted into a fraction other than the non-saponifiable residue during the course of saponification. The other behaves in an orthodox way. Whether this latter factor is to be identified with the vitamin D of cod-liver oil, whether it is the same as irradiated ergosterol, or whether, possibly, it differs from those two recognised factors, we are at present obviously not in a position to say. We only know that it shares with those two factors their resistance to saponification. It has been so far our experience that, by whichever method we

attempted to increase the antirachitic activity of the butter, the added factor behaved in a normal way.

It would therefore appear that while the cow possesses the ability of secreting into her milk not only vitamin D but also another substance or property antirachitically active for the rat, the yield of this substance cannot be influenced either by changes in the natural conditions such as changes from winter to summer types of feeding, or by massive feeding of highly potent carriers of vitamin D activity, or, lastly by direct irradiation of the butter. The cow thus appears to be able to produce a novel antirachitic agent, but definitely does not rely for its production upon any exogenous source of vitamin D *sensu stricto*, whatever this term implies. A physiological conversion of added known forms of vitamin D into the novel form found in butter has not, in our experience, been found to occur.

As far as we can judge, this factor would seem constant in all the butters and always present in the same amount in a given bulk of butter. Thus, for example, it might constitute 75 % of the antirachitic activity of a butter of which 1 g. daily is required to give almost complete protection. When on the other hand the activity of such a butter is raised nearly a hundred times and 10 mg. of this butter nearly suffice to afford complete protection, the orthodox factor only is increased and the rôle played by the unresistant factor becomes so small that its loss on saponification is not noticeable.

Summary.

1. The following butters have been tested for their antirachitic potency both before and after saponification: (*a*) butter from cows on summer pasture, (*b*) butters from cows having received two different levels of irradiated yeast, (*c*) butter from cows having received cod-liver oil, (*d*) irradiated winter butter. It was found that the antirachitic value of high-level yeast butter and of irradiated butter could be recovered practically completely in the non-saponifiable residue, while the remaining butters suffered a more or less marked loss in the course of saponification. These butters, however, including summer butter, were definitely more resistant to saponification than the autumn and winter butters reported on in the previous paper of this series.

2. In confirmation of previous work it was found that a large part (over 75 %) of the potency of autumn and winter butters was either destroyed or diverted to another fraction on preparation of the non-saponifiable residues.

3. The greater the initial antirachitic potency of the butter, the greater is the proportion of the potency recoverable in the non-saponifiable residue.

4. It is suggested that at least two factors antirachitic for the rat are present in butter; a factor which is not recoverable in the non-saponifiable residue, and the usual vitamin D (whatever this term may imply), which is resistant to saponification. By exposure to summer conditions or by the feeding to cows of highly antirachitic substances such as irradiated yeast or cod-liver oil the concentration of the stable factor but not of the labile factor of butter can be markedly increased. Direct irradiation of the butter also leads to the formation of the stable factor only.

5. Attempts to achieve a chemical separation of the labile and stable antirachitic factors of butter by submitting butters of varying potency to extraction with alcohol, following the method of Zucker, failed in so far as we were unable, contrary to Zucker's findings, to extract by this method the antirachitic potency of a high grade cod-liver oil, or, in agreement with Zucker, to extract that of butter.

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REFERENCES.

Golding, Mackintosh and Mattick (1932). *J. Dairy Res.* **4**, 48.
Kon and Booth (1933, 1). *Chem. Ind.* **52**, 420.
—— —— (1933, 2). *Biochem. J.* **27**, 1302.
Shipley, Kinney and McCollum (1924). *J. Biol. Chem.* **59**, 177.
Steenbock and Wirick (1930). *J. Dairy Sci.* **13**, 497.
Zucker (1922–23). *Proc. Soc. Exp. Biol. Med.* **20**, 136.
—— (1925). Brit. Patent No. 30362/23.
—— (1928). U.S. Patent, 1,678,454.
—— (1933, 1). *Science*, **77**, 19.
—— (1933, 2). *Amer. J. Public Health*, **23**, 10.
—— and Barnett (1922–23). *Proc. Soc. Exp. Biol. Med.* **20**, 375.

XVIII. THE VITAMIN D ACTIVITY OF BUTTER.

III. AN ATTEMPT TO ELUCIDATE THE NATURE OF THE LABILE FACTOR IN BUTTER ANTIRACHITIC FOR THE RAT. THE ANTIRACHITIC POTENCY OF LARD, OLIVE OIL, EGG OIL AND THE FATTY ACIDS OF BUTTERS AND LARD.

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In the previous paper of this series [Kon and Booth, 1934] we have summarised the evidence which points to the existence in butter of two factors antirachitic for the rat, the differentiation between the two factors being based upon the inability to recover one of them in the non-saponifiable residue of butter.

The present paper deals with a description of our efforts to elucidate the nature of the labile factor.

Our experiments were carried out in three main directions.

1. Firstly, we thought it advisable to investigate the effect of saponification on the antirachitic potency of eggs. With the exception of fish-liver oils and of some oils of marine animals, egg oil is probably the most concentrated natural source of vitamin D activity. Excluding such additions to the diet as cod-liver oil and fish meal it would seem that the exogenous sources of the antirachitic factor, or factors, available to the laying hen kept on open range are very similar to those placed at the disposal of the cow. Exposure to sunshine is common to both, as is also the consumption of grass, though in the latter case obviously in different relative quantities. We know of no marked difference in the qualitative or quantitative sense between the vitamin D activity, if any, of the other basal vegetable constituents of the diet of either the hen or the cow.

We therefore studied the effect of saponification on the antirachitic activity of eggs obtained from hens kept on open range and receiving no addition of either fish meal or cod-liver oil to their diet.

2. Secondly, we examined the fatty acid fraction of butters for antirachitic action.

Some ten years ago, Zucker and Barnett [1922–23], in trying to explain their failure to concentrate the vitamin D activity of butter in a manner successfully used by them in the preparation of an active concentrate from cod-liver oil, advanced the hypothesis that the antirachitic activity of butter as measured on the rat might be largely, if not solely, due to the action of the fatty acids of butter combining with the dietary calcium and causing the excretion of increased amounts of calcium from the gut in the form of calcium soaps. While we do not necessarily agree with Zucker and Barnett's explanation of the mechanism of fat action, we were nevertheless impressed, as they were, by the possibility of the saponifiable fraction of butter playing a rôle in the total antirachitic activity

of the latter. We have therefore planned and carried out experiments which, in our opinion, might help in elucidating the problem.

Zucker and Barnett's suggestion, as far as we understand it, was made in an attempt to reconcile the success of their method of concentrating the antirachitic factor of cod-liver oil with the failure of this method as applied to butter and other substances. It was not based on experimental evidence derived from the feeding to rats of the fatty acids of either butter or of the vegetable fats mentioned in their paper. We [Kon and Booth, 1934] have not only failed to extract by the above method the antirachitic activity of butter, but were equally unsuccessful in the case of cod-liver oil. On this basis, therefore, we see no *a priori* reason for ascribing the antirachitic activity of butter, partly or wholly, to its fatty acid moiety. Nevertheless, the peculiarities of the etiology of experimental rickets in rats make it imperative to investigate experimentally the possible rôle of the fatty acid fraction of butter. The rachitic condition in the rat cannot be brought about by a deficiency of vitamin D alone. A rachitogenic diet must also be grossly abnormal in the proportion of its mineral constituents in order to produce a good degree of rickets. It is well known [Karelitz and Shohl, 1927; Brown *et al.*, 1932; Shohl *et al.*, 1932; Key and Morgan, 1932] that the mere addition of phosphorus to a rachitogenic diet, or the withdrawal of a part of the calcium, produces the same effect as if an antirachitic substance had been added to the otherwise unchanged diet. It is, therefore, evident that any substance which would alter the calcium and phosphorus metabolism of the rachitic rat, either by increasing the excretion of calcium or by facilitating the uptake of phosphate from the gut, will behave, in bringing about a prevention or cure of the rachitic condition, in a manner indistinguishable from that of a true antirachitic agent.

The smaller the original potency of a butter the larger is the amount which it is necessary to feed to ascertain its antirachitic activity. It should be remembered that, as the fatty acids of butter weigh almost as much as butter itself, the amounts of fatty acids which it is necessary to administer to the rat when testing butters of low or moderate activity are quite considerable. There is ample evidence which has been lately summarised by Boyd *et al.* [1932] that fatty acids play a very definite rôle in the metabolism of calcium and phosphorus in the rat.

Under the second heading also must come our experiments in which we fed to rats the non-saponifiable residue simultaneously with the fatty acid fraction of butter. If that part of the activity of butter which is not recoverable after saponification is due to the antirachitic action of the fat, then such a "reconstituted" butter should regain its original activity.

We found it impossible to feed fatty acids separately from the diet by pipette as we feed butters. The higher melting-point formed one obstacle. A more serious one was the refusal of the rats to consume the fatty acids when given by pipette. These had therefore to be fed incorporated in the diet and as a check we thought it desirable to feed butter likewise incorporated in the diet.

3. Lastly, we fed to rats a vegetable fat, olive oil, and an animal fat, lard, in quantities comparable with those administered in the case of butters of low or moderate antirachitic activity.

Olive oil is generally recommended for use and is used as a diluent for cod-liver oil and a solvent for irradiated ergosterol. In small quantities high grade olive oil which has not been exposed to sunlight is antirachitically quite inert. The effects, if any, of large quantities of this fat would therefore, in our opinion, throw a valuable light on the fatty acid hypothesis of the antirachitic activity of butter.

The use of lard as a constituent of a rachitogenic diet composed mainly of purified foodstuffs has been suggested by Osborne and Mendel [1923] and Smith and Mendel [1930]. These authors advise the use of diets containing lactalbumin or edestin, starch, a phosphorus-free salt mixture and from 0 to 24 % lard. For this reason we chose lard as a representative animal fat which might be considered to be antirachitically inert.

Experimental.

Methods.

1. *Egg oil.* Fresh eggs were obtained at the end of June 1933 from Light Sussex hens on open range and receiving a grain mixture containing meat meal but no cod-liver oil or fish meal. Eggs were also collected at the end of October 1933 from the same hens receiving the same diet.

The yolks of the eggs were carefully separated from the whites, and after adding an equal volume of 8 % NaCl [Jukes and Kay, 1932] they were extracted three times with ether, the third extract being almost colourless. The extract was washed twice with water, dried over anhydrous Na_2SO_4 and the ether evaporated after filtering. For feeding, the egg oil was diluted with olive oil in the usual way. After saponification of 10 g. of egg oil the mixture of soaps was extracted seven times with ether using one portion of 75 ml. and six portions of 50 ml. This number of extractions was necessary in order to remove the bulk of the colouring matter (xanthophyll). It is very probable that a smaller number of extractions would have effected a quantitative recovery of the antirachitic factor. The non-saponifiable residue was dissolved in olive oil for feeding as previously described.

2. *Fatty acids.* All the fatty acids discussed below were prepared by the following method. After removal of the non-saponifiable residue from saponified butter or lard the soaps were acidified with sulphuric acid and the fatty acids extracted three times with ether. The ethereal solution was washed twice with water and dried over anhydrous Na_2SO_4. The ether was then removed, firstly on the water-bath and then under diminished pressure. This method of recovery is not quite quantitative, losses occurring in the lower volatile and water-soluble fatty acids. For feeding, the fatty acids were incorporated in diet 2965 of Steenbock by dissolving them in ether, mixing the solution with the diet and subsequently allowing the solvent to evaporate. The fatty acids derived from the following butters and lard were prepared and fed[1]: summer butter; irradiated butter; autumn butter; high-level yeast butter; winter butter; home-rendered lard. Records of food intake of the several groups of rats receiving these fatty acids were kept. As in the case of all other substances tested, each fatty acid and level of fatty acid was fed to groups of 4 rats kept in one cage. The food records give therefore the average amount of fatty acid consumed by any rat in the group. It will be remembered [Kon and Booth, 1933; 1934] that all substances fed separately from the diet were administered on weekdays only, while the substances incorporated in the diet were given seven times a week. Therefore to give a true impression of the antirachitic activity, if any, of the fatty acids we give in the following table average weekly consumptions. The fatty acids were usually fed in the same experiments as those involving the feeding of the corresponding original fats and their non-saponifiable residues. Whenever several samples of butter were saponified in the course of one feeding test, fresh

[1] The corresponding butters were discussed in our previous paper [Kon and Booth, 1934].

samples of the corresponding fatty acid-containing diets were prepared on the same days as those on which the changes were made in the feeding of the fats and non-saponifiable residues.

When fatty acids were fed to rats simultaneously with the administration of the non-saponifiable residues, the fatty acids were fed exactly as described above, and the non-saponifiable residues were administered six times a week in the usual way.

For feeding in the diet the butter-fat was melted and poured over the requisite amount of the 2965 rachitogenic diet and was then well kneaded in by hand until a perfectly homogeneous material was obtained. The bulk of this was stored in a cold place. These diets were fed and food intake was recorded in the same way as in the case of fatty acids.

3. (*a*) *Olive oil.* The olive oil was fed by pipette separately from the diet in an exactly similar manner to the butters. It was a high grade first pressing Italian oil which had been used by us with complete satisfaction as a diluent for cod-liver oil and the International Standard of Vitamin D, and as a solvent for the various non-saponifiable residues and other substances requiring dilution.

(*b*) *Lard.* Three samples of lard were tested, two of them were purchased from a local grocer and were both of American origin. It is quite possible that they may have been both from the same consignment, but we prefer to consider these lards as separate specimens. The second sample was from a bulk labelled: "White Rose Pure Lard, Dold Quality, Buffalo, U.S.A."

The third sample was a lard rendered by us from the leaf fat of 4 pigs slaughtered at bacon weight at the end of September 1933 when 7 months old. For the last 4 months of life they had been kept indoors without access to sunlight and grass and had been maintained on the following diet:

Barley meal	48·0 %	Middlings (Weatings)	35·0 %
Soya-bean meal	6·0	Meat meal	4·0
Dried whole milk	5·0	Ground limestone	1·75
	Sodium chloride	0·25 %	

It is reasonable to suppose that the vitamin D reserves, if any, of these animals were of a low order. For rendering, the chopped fat was heated in a pail immersed in boiling water and the fat filtered through cheese-cloth, allowed to settle and set, remelted and filtered through a fluted filter.

The first sample of lard was fed only as such. The second and third samples were fed both before and after saponification. The non-saponifiable residues were extracted four times with ether. The lards were given by pipette separately from the diet. One level of the first sample was fed mixed in the diet.

It will be seen from Table I that there is no difference between the anti-rachitic effects of doses of summer egg oil and those of corresponding levels of the non-saponifiable residues of the same oils. On the other hand the highest of the three levels tested did not afford more than a slight degree of protection. A reference to the previous paper of this series [Kon and Booth, 1934] will show that, although in the case of summer butter and of cod-liver oil butter there was no difference between the antirachitic effects of the butter and of its non-saponifiable residue at the lowest level of intake, which gave an ash percentage similar to that produced by the highest level of egg oil, definite differences could be noted at the higher levels. In the light of our experience with summer egg oil the levels of autumn egg oil were better selected, but unfortunately an error was made in the dilution of the non-saponifiable residue which was made up to twice

Table I. *Summer and autumn 1933. Prophylactic experiments. Antirachitic effect of various egg oils, lards, butters and their non-saponifiable residues; olive oil; fatty acids of lard and butters; fatty acids and non-saponifiable residue of butter fed together; butter incorporated in the diet. Percentage of ash in dry defatted femora and humeri.*

The figures for ash percentages are averages of duplicate determinations on groups of 4 rats.
The dosages of non-saponifiable residues are expressed in equivalents of the butter or lard from which they were derived.

Substance tested	Dosage g.	Ash %
Summer egg oil	0·001	30·5
Summer egg oil	0·003	34·1
Summer egg oil	0·006	38·9
Non-saponifiable residue of summer egg oil, equiv. ...	0·001	30·1
Non-saponifiable residue of summer egg oil, equiv. ...	0·003	34·0
Non-saponifiable residue of summer egg oil, equiv. ...	0·006	38·2
Autumn egg oil	0·0115	41·2
Autumn egg oil	0·0345	51·5
Non-saponifiable residue of autumn egg oil, equiv. ...	0·0230	42·2
Non-saponifiable residue of autumn egg oil, equiv. ...	0·0690	52·0
Olive oil	1·0	37·4
Negative controls for olive oil	—	30·1
American lard, 1st sample	0·25	43·9
American lard, 1st sample	0·50	51·2
American lard, 1st sample	1·00	51·5
American lard, 1st sample incorporated in the diet, approximately	1·50	49·2
American lard, 2nd sample	0·5	48·5
American lard, 2nd sample	1·0	51·3
Non-saponifiable residue of American lard, 2nd sample, equiv.	0·5	41·5
Non-saponifiable residue of American lard, 2nd sample, equiv.	1·0	45·4
Home-rendered lard	1·0	48·0
Non-saponifiable residue of home rendered lard, equiv.	1·0	34·5
Negative controls for American lard, 2nd sample and for home-rendered lard	—	34·9

Substance tested	% in diet	Av. weekly intake of substance per rat g.	Ash %
Summer butter	Fed separately	3·0	49·5
Summer butter fatty acids	6·25	3·0	37·6
Negative controls at this time	—	—	32·0
Irradiated butter	Fed separately	0·06	46·9
Irradiated butter fatty acids	0·20	0·15	30·9
Winter butter	Fed separately	4·20	47·3
Winter butter fatty acids	7·00	3·23	35·8
Negative controls for irradiated butter and winter butter	—	—	34·9
Autumn butter	Fed separately	3·00	47·1
Non-saponifiable residue of autumn butter	Fed separately, equiv.	3·00	39·1
Autumn butter fatty acids	6·25	3·26	36·7
Autumn butter fatty acids + non-saponifiable residue	6·25	3·37	41·2
Autumn butter in diet	6·25	3·78	43·3
Autumn butter	Fed separately	6·00	52·9
Non-saponifiable residue of autumn butter	Fed separately, equiv.	6·00	40·3
Autumn butter fatty acids	12·50	6·16	40·3
Autumn butter fatty acids + N.S.R.	12·50	5·75	50·2
Autumn butter in diet	12·50	6·80	48·5
Negative controls for autumn butter	—	—	33·2
High-level yeast butter	Fed separately	0·06	46·0
High-level yeast butter fatty acids	1·25	0·75	43·1
Negative controls for high level yeast butter	—	—	32·0
Home-rendered lard fatty acids	12·50	5·60	39·7
Negative controls for these fatty acids	—	—	33·2

the concentration of the original oil. The results might be interpreted as indicating a certain loss of the antirachitic potency during the course of saponification. We would prefer for the time, however, to consider these figures as inconclusive. It should be mentioned here that Hess and Weinstock [1924] found that the non-saponifiable residue of egg-yolk was antirachitically active and obtained a preparation about 90 times as concentrated as the original yolk. We are planning to investigate the effect of saponification on eggs from the same source as those obtained and tested by us in summer and autumn, but collected in February when their antirachitic potency should be at its lowest.

The feeding of the olive oil at a 1 g. level had a definite effect upon the severity of the rachitic condition. It will be noticed that the bones of rats receiving this quantity of olive oil contained 7 % more ash than those of the negative controls.

The results of feeding the first sample of the American lard were quite startling. The antirachitic effect of this lard was, if anything, better than that of the summer butter fed at the same time. (The figures for this butter are given in the previous paper [Kon and Booth, 1934].) No records of food consumption were kept in the case of lard incorporated in the diet, but an average consumption of 8 g. per day would imply the ingestion of 1·5 g. of lard. It would appear that lard incorporated in the diet is less antirachitically effective than when administered separately from it.

The second sample of American lard was also, comparatively speaking, very potent and gave results similar to those produced by the first sample. The non-saponifiable residue gave definitely lower values than the lard itself, and the degree of loss was very similar to that which occurred in the case of summer butter.

The home-rendered lard was less potent than the two American lards. Very marked loss took place here after saponification, the rats receiving the non-saponifiable residue being indistinguishable from negative controls.

Drummond *et al.* [1920], in a paper written before vitamins A and D were differentiated, stated that the addition of lard obtained from pigs receiving an ample supply of the fat-soluble vitamin definitely increased the growth and improved the condition of rats kept on a diet deficient in this factor, while lard from a pig without such supply of vitamin failed to do so. Quite recently (since this work was started) Read and Bailey [1933] have stated that some lards may contain an appreciable amount of vitamin D, while others contain an inappreciable amount of this factor.

On reviewing the antirachitic effect of fatty acids it is apparent that their action, though slight, is unmistakable. With the sole exception of those of high-level yeast butter the fatty acids, even at a level of 1 g. daily, do not increase the ash beyond 40 %. The case of the high-level yeast butter fatty acids requires some explanation. These fatty acids fed at a level of approximately 0·12 g. daily produced an ash percentage of 43·1, quite definitely higher than that given at the same time by the negative controls. On the other hand the degree of protection afforded by this amount of fatty acid is lower than that given by one-twelfth this amount of the original butter. This was the only fatty acid of this series which was separated from the non-saponifiable residue by only three ether extractions. If only 5 % of the non-saponifiable residue were left in the fatty acids, which under these circumstances is quite possible, the activity of this sample of fatty acids would be accounted for.

In the case of the remaining fatty acids which had all been separated from their non-saponifiable residues by four or, in the case of autumn butter, five extrac-

tions, no activity ascribable to the non-saponifiable fraction would seem to be left in the acids. The activity of the acids does not seem to bear any relation to the original total activity of the fat but appears to be proportional to the amount of fatty acid consumed. Thus for example the fatty acids of irradiated butter, though fed at almost three times the level of the original irradiated butter, gave an ash percentage actually lower than that given by the negative controls for this group of experiments, though the irradiated butter fed at the lower level afforded almost complete protection.

The combination of fatty acids and non-saponifiable residue of autumn butter gave, at the higher level of 1 g., only a slightly lower value than that produced by 1 g. of untreated butter. On the other hand the combination of fatty acid and non-saponifiable residue proved definitely less effective at the lower level of 0·5 g. than the corresponding amount of untreated butter.

When the butter was incorporated in the diet instead of being fed separately from it, the ash percentages were definitely lower in the former than in the latter case, although on the basis of food consumption slightly more butter was consumed by the animals which received it as an admixture to the diet. As already stated we noticed this phenomenon also in the case of lard.

Discussion.

In order to appraise the significance of the experimental results described in this paper the following points should, in our opinion, be taken into consideration.

Firstly, with regard to its antirachitic properties lard behaves in a way very similar to that of butter. Different samples of lard display activities ranging from that of winter to that of summer butter, the antirachitic potency being lost on saponification in a way closely resembling that found in the case of butter, the loss being less marked in the more potent sample.

Secondly, when fed at 0·5 and 1·0 g. levels all fatty acids seem to have had a definitely measurable antirachitic effect. It seems extremely doubtful whether this effect can be attributed to incomplete removal of the stable vitamin D, as the amount of total activity which would have to remain in the fatty acids in order to produce such an effect would be far too great in the case of butters of originally low activity. Even on the unlikely assumption that, after 4 or 5 extractions with ether, 5 % of the orthodox activity had been left in the fatty acids, the amount so left in the case of autumn butter or of home-rendered lard would be quite insufficient to raise the ash percentage so definitely above the level of the negative controls. The completely negative results in the case of the fatty acids of irradiated butter, which were fed at a level relatively (on the basis of original potency) more than double that of the fatty acids of the butters and lard of low activity, also point in the same direction.

Thirdly, when butter is incorporated in the diet, its antirachitic effect is definitely lower than that of an equivalent amount of butter fed separately. The same phenomenon can also be noticed in the case of lard. It should be emphasised here that this difference in potency according to the method of feeding cannot in itself explain or account for the very marked drop in potency caused by saponification. A comparison of the ash percentages produced by 0·5 and 1·0 g. of autumn butter fed in the diet with those for corresponding levels of the same butter after saponification given in Table I bears out this statement. As already mentioned we have been technically unable to feed the fatty acids separately from the diet. Should they behave in a way similar to that of the original butter, then their antirachitic effect would be more marked when fed separately.

Fourthly, a combination of the fatty acids and non-saponifiable residue of autumn butter elicits an antirachitic effect superior to that produced by either component fed separately. As the fatty acids were fed in the diet we think that the antirachitic effect of such a "reconstituted" butter should be compared with that of corresponding amounts of the original butter also incorporated in the diet. When this is done it will be found that the difference in antirachitic effect is so slight as to be well within the limits of experimental error. In other words, it would seem that the total activity of butter (of this particular sample of autumn butter in any case) can be recovered quantitatively after saponification when the fatty acid moiety of the butter is recombined with the non-saponifiable fraction.

We have proved to our entire satisfaction in the two previous papers of this series [Kon and Booth, 1933; 1934] that the loss of potency following saponification evident in certain butters cannot be attributed to incomplete extraction of the non-saponifiable residue. Bearing this in mind and adding the evidence just discussed we can arrive at the following alternative suggestions as to the nature of the labile antirachitic factor of butter.

A. The first possibility would be to accept the existence in butter and in lard of a novel antirachitic factor unstable to saponification, and in general more labile than the usual vitamin D. This factor would appear to lose a part of its potency immediately after the butter or lard is mixed with the 2965 rachitogenic diet even when it is kept thereafter at a fairly low temperature; conditions under which irradiated ergosterol and cod-liver oil are stable for longer periods. The existence of such a factor seems quite conceivable were it not for the fact that it is difficult to account on this basis for the slight but unmistakable residual activity of the fatty acids. Also, if the existence of the novel factor should be the true explanation, it is very difficult to understand why a combination of fatty acids and non-saponifiable residue should have virtually the same antirachitic activity as corresponding levels of butter incorporated in the rachitogenic diet.

B. It might also be suggested that the antirachitic activity of butter was the resultant of two factors, namely, the activity of vitamin D *sensu stricto*, whatever this term implies, and of the antirachitic action of fat. In other words it might be suggested that the rat gives, because of its physiological peculiarities, an enlarged and distorted picture of the "true" antirachitic activity of butter, reflecting not only a real antirachitic factor pharmacologically active in different species, but also a non-specific stimulus antirachitic only in the narrow "rat" sense of the word. There is much in our experimental evidence in favour of such a view. The residual activity of the fatty acids, remarkably constant in the case of butters and lard and independent of the activity of the parent substance, and the possibility of reconstructing the original activity of butter by combining fatty acids and non-saponifiable residue speak strongly in favour of such an hypothesis. This explanation has also in its favour the argument of simplicity, as it avoids the necessity of adding another general antirachitic factor to the two already existing. It explains without difficulty why lard should behave in a similar way to butter and also accounts for the slight activities of various vegetable oils reported in the literature and found by us in the case of olive oil.

If also that part of the antirachitic activity of butter which cannot be recovered after saponification is not due to the presence of a specific antirachitic factor but to the action of fat, it is obvious that any attempt to increase the vitamin D activity of butter [Kon and Booth, 1934] would result only in an

increase in the orthodox vitamin D and not in the novel factor. This, in our experience [Kon and Booth, 1934] is actually the case.

Our finding that either butter or lard incorporated in the diet is less active than corresponding levels fed separately is not incompatible with the suggestion that a part of the antirachitic activity of these substances is due to their fatty acids. The antirachitic action of fat in the case of the rat must be due to the influence of the fat on the calcium and phosphorus metabolism, and it seems quite permissible to assume that the effect of one massive dose daily will be different from that of the same amount distributed over a period of 24 hours.

We have already mentioned that Zucker and Barnett [1922–23] suggest as an explanation of the mechanism of the action of fat the formation of insoluble calcium soaps. Recent work [Boyd *et al.*, 1932] would rather favour the view that the beneficial effect of fat is due to the creation in the gut of a p_H favourable to the uptake of minerals. We do not want to theorise on this point but wish to postpone judgment pending the collection by us of experimental data on the effect of various fatty acids on the calcium and phosphorus metabolism of the rachitic rat.

It would seem to us, that on the whole the arguments in favour of fat playing a rôle in the antirachitic activity of butter are quite serious enough to deserve careful consideration.

C. There is just a possibility that both fat and a novel factor are responsible for the antirachitic activity of the labile factor of butter and lard in the case of the rat. Insufficient experimental evidence prevents us at present from discussing this point any further.

We are led to believe that, unless the labile factor of butter is definitely proved to be antirachitic also for other species besides the rat, the only safe course for ascertaining the vitamin D activity of butter is to submit it to saponification and feed to rats the non-saponifiable residue only. Even then in order to decide *a priori* what might be the antirachitic value of such butter in human nutrition, it would be necessary to find out whether the orthodox antirachitic factor present therein is identical with irradiated ergosterol or with the vitamin D of cod-liver oil, or whether it is altogether different from these two.

We [Kon and Booth, 1933; 1934] have examined various butters by the widely used prophylactic method, making use of what is practically the official diet (the 2965 diet of Steenbock) and administering graded doses of butter separately from the diet, a method used to our knowledge by the majority of present day workers. We have very good reason to believe that, in the case of many butters, especially those collected in autumn and winter, values so found by this generally used technique for their alleged vitamin D content may be anything up to 4 or 5 times too high.

It may be also remarked that similar results were obtained by the line test technique as far as this is possible with butters of low potency [Kon and Booth, 1933; 1934].

We have purposely refrained from any discussion of the clinical antirachitic value of butter, but we think that our results may perhaps suggest a plausible explanation of the discrepancy of opinion existing between the clinician and the student of nutrition with regard to the antirachitic value of butter.

Summary.

1. When tested on rats, lard was found to possess an antirachitic activity of the same order as that of butter, a sample of home rendered lard being almost equal in that respect to winter and autumn butter, while a sample of American

lard was found to be fully as active as summer butter. As in the case of butter, the activity of lard is impaired by saponification, the degree of loss being greater in the case of the lard of lower activity.

2. Fatty acids prepared from various butters were found to exhibit a slight but definite antirachitic activity which could not be attributed to incomplete removal of the non-saponifiable residue. This activity was roughly proportional to the amount of fatty acid fed and was independent of the original activity of the butter.

3. When butter was incorporated in the diet, it was found to produce a lower antirachitic effect than that given by a similar amount of butter fed separately from the diet.

4. In the case of an autumn butter, which after saponification had lost a large part of its original antirachitic potency, this potency could be recovered practically completely when the administration of the non-saponifiable residue was accompanied by the feeding of the fatty acid incorporated in the diet.

5. Although the loss of potency observed by us in various samples of butter could be tentatively explained by assuming the existence of a new labile antirachitic factor, we are inclined to believe at present that this phenomenon could be at least as adequately, and certainly more simply, explained by the antirachitic effect on the rat of fat as such.

6. If this explanation is, as we believe, correct, then, unless the labile factor of butter is definitely proved to be antirachitic also for other species besides the rat, values obtained by accepted methods for the antirachitic activities of butters are about 4 to 5 times too high in winter and autumn and about twice too high in summer. On the other hand values obtained by direct feeding of highly potent butters giving complete protection at a daily level of 10–30 mg., such as irradiated butter or butter yielded by cows receiving large amounts of irradiated yeast, are correct.

7. On the same basis it is suggested that while the general antirachitic value of the labile factor is still in question the only safe course in ascertaining the vitamin D activity of butter is to submit it to saponification and feed to rats the non-saponifiable residue only.

We are indebted to Capt. J. Golding and Prof. H. D. Kay for helpful criticism; to the former also for the home-rendered lard; to Miss D. V. Dearden for the churning of the butters and to Mrs S. K. Kon for much help in the preparation of bones for analysis.

REFERENCES.

Boyd, Crum and Lyman (1932). *J. Biol. Chem.* **95**, 29.
Brown, Shohl, Chapman, Rose and Saurwein (1932). *J. Biol. Chem.* **98**, 207.
Drummond, Golding, Zilva and Coward (1920). *Biochem. J.* **14**, 742.
Hess and Weinstock (1924). *Proc. Soc. Exp. Biol. Med.* **21**, 441.
Jukes and Kay (1932). *J. Exp. Med.* **56**, 469.
Karelitz and Shohl (1927). *J. Biol. Chem.* **73**, 665.
Key and Morgan (1932). *Biochem. J.* **26**, 196.
Kon and Booth (1933). *Biochem. J.* **27**, 1302.
—— —— (1934). *Biochem. J.* **28**, 111.
Osborne and Mendel (1923). *Proc. Soc. Exp. Biol. Med.* **21**, 87.
Read and Bailey (1933). *Cereal Chem.* **10**, 99.
Shohl, Brown, Chapman, Rose and Saurwein (1932). *J. Biol. Chem.* **98**, 215.
Smith and Mendel (1930). Article in Abderhalden's Handbuch der biologischen Arbeitsmethoden, Abt. IV. Teil 13, 117. (Urban and Schwarzenberg, Berlin and Vienna.)
Zucker and Barnett (1922–23). *Proc. Soc. Exp. Biol. Med.* **20**, 375.

XIX. THE BIOCHEMISTRY AND PHYSIOLOGY OF GLUCURONIC ACID.

IV. (*a*) THE OCCURRENCE OF CONJUGATED GLUCURONIC ACIDS IN THE ANIMAL BODY.

(*b*) OBSERVATIONS ON THE CONJUGATION OF *d*- AND *l*-BORNEOL.

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Glucuronic acid in normal animal urine.

Following the recognition of the part played by glucuronic acid in detoxication mechanisms, its possible occurrence in normal urine was suggested by Hoppe-Seyler [1882] and was further considered by Flückiger [1885] in the course of his investigations of the copper-reducing substances of human and animal urines. Several early observers [Haas, 1876 and others] had shown that normal human urine is slightly laevorotatory and it was probable that this laevorotation was due to the presence of conjugated glucuronic acids, which are all laevorotatory. Proof of the occurrence of glucuronic acid in normal urine was provided by Mayer and Neuberg [1900], who isolated it from human urine as the *p*-bromophenylhydrazine derivative. These workers concluded that glucuronic acid occurs in normal human urine conjugated mainly with phenol and cresol and to a lesser extent with indoxyl and skatoxyl. It had previously been suggested by Hoppe-Seyler [1882] that glucuronic acid might be excreted in normal urine as the conjugated indoxyl compound, and Mester [1888] found a laevorotatory and copper-reducing substance in the urine of dogs after administration of skatole, which he regarded as the hypothetical skatoxylglucuronic acid. Neuberg and Niemann [1905] also suggested that urea-glucuronic acid, which they had synthesised, might occur in normal urine, and Pollecoff [1924] stated that glucuronic acid is a probable constituent of urochrome, the yellow pigment of urine, isolated from human urine and carefully purified. Tollens [1910] has emphasised the fact that man excretes twice as much conjugated glucuronic acid daily as ethereal sulphate and even within the limits of normal behaviour much more glucuronic acid can be excreted daily than of ethereal sulphate. The quantity of glucuronic acid in normal urine has been determined by several workers and values from 4 to 25 mg. per 100 ml. have been quoted (see Table I).

With reference to the occurrence of glucuronic acid in the urine of other animals little can be found in the literature. Hagemann [1882] states that it is probably a part of the reducing substance he obtained from horse urine, while Kawanishi [1932] has estimated it in rabbit urine (see Table I). We have detected

Table I.

Observers	mg. glucuronic acid per 100 ml. urine: Normal human urine	Rabbit urine
Boku and Kin [1931]	13	—
Kawanishi [1932]	5 to 10	4 to 15
Mayer and Neuberg [1900]	4	—
Sauer [1930]	15 to 19	—
Tollens and Stern [1910]	25	—

glucuronic acid in the normal urine of all animals tested, using the Tollens naphthoresorcinol test, and we have confirmed its presence in human urine by the same test (see Table II).

Table II.

Animal	No. tested	Naphthoresorcinol test
Man	15	+
Dog	30	+
Cat	18	+ + +
Mouse	6	+ +
Guinea-pig	2	+

It is interesting to note that cat urines always give a much stronger positive test than the urine of other animals listed, and this strong test is associated with an intense Obermayer indoxyl reaction. It would seem probable that cat urine normally contains a relatively greater amount of the hitherto undescribed indoxylglucuronic acid than the urine of other animals tested, and it is therefore a likely source for isolating this compound. The conjugated glucuronic acids of normal urine (*i.e.* phenyl, cresyl, indoxyl and skatoxyl) have not been isolated from such urine, but phenylglucuronic acid has been isolated from the urine of sheep after administration of phenol [Salkowski and Neuberg, 1906]; cresylglucuronic acid has been isolated as a double barium salt with phenylsulphuric acid from the urine of dogs fed with *p*-cresol [Neuberg and Kretschmer, 1911]; and Neuberg and Schwenk [1917] have isolated a double barium salt of indoxylglucuronic and -sulphuric acids from urine after the feeding of indole. It is of interest to point out that the non-fermentable sugar of urine, which was shown by West *et al.* [1932] to increase during intestinal stasis in the dog, is probably in part glucuronic acid, which itself is not fermented by yeast.

Glucuronic acid in normal animal blood.

Scanty reference has been made in the literature to the occurrence of glucuronic acid derivatives in blood, but their presence there follows from their elimination by the kidney, since it is shown that the kidney does not synthesise conjugated glucuronic acids [Hemingway *et al.*, 1934]. Glucuronic acid, as the *p*-bromophenylhydrazine derivative, has been isolated from bovine blood by Mayer [1901], who states that it is probably constantly circulating in human blood. Letsche [1907] states that amongst the reducing substances in blood of carbohydrate nature are glucose and glucuronic acid; the presence of the latter was deduced from orcinol- and phloroglucinol-HCl tests and from the isolation of a hydrazone. Stepp [1919] showed it to be present in normal human blood by means of the naphthoresorcinol test, and Ørskov [1932], in a study of the ether-soluble acids of blood, claims that minute quantities of conjugated glucuronic acids are present. Lépine and Boulud [1905; 1906] have made estimations of the conjugated glucuronic acid in arterial and venous blood, in serum

and in the corpuscles, and find as much as 14 to 47 % of the blood-sugar to be conjugated glucuronic acids; they base their values on reducing powers and rotations, but, owing to interfering substances (now known to be present in blood) which affect these methods, these values cannot be accepted without further investigation.

We have confirmed the fact that human blood contains glucuronic acid, using the naphthoresorcinol reaction and find that dog and cat bloods also give a slight positive test. The test was carried out on blood after deproteinising with sodium tungstate and sulphuric acid. The test was then compared with a blank using the deproteinising agents and water and with a known positive test on blood containing bornylglucuronic acid. We have also found that the blood

Table III.

Animal	Method	Observer
Man	Naphthoresorcinol test	Stepp [1919] and this investigation
Dog	,,	This investigation
Cat	,,	,,
Ox	Isolation of *p*-bromophenylhydrazide	Mayer [1901]

and serum of man and the dog give very intense naphthoresorcinol tests for glucuronic acid, 10 minutes after oral administration of 2 g. of borneol, from which it seems that the detoxication of borneol begins to take place rapidly.

The stereochemistry of bornylglucuronide in relation to its formation in the body.

All natural glycosides possess the β-type of linkage, and this may now be regarded as true also for glucuronides synthesised *in vivo*. Bornylglucuronide, from the urine of dogs fed with borneol, has been shown by Hämäläienen [1911] to be a β-glucosidic compound, since it is hydrolysed by the β-glucosidase of emulsin. However, Miwa [1932] stated that not all β-glucuronides are hydrolysed by this β-glucosidase. Positive results have been obtained by Neuberg and Niemann [1905] with phenylglucuronide and euxanthic acid, by Hämäläienen [1911] with *l*-camphorylglucuronide, by Hildebrandt [1905] with syringa- and vanillin-glucuronide, by da Cruz [1930] with menthylglucuronide, and by Ishidate [1929] with *d*-camphorylglucuronide and menthylglucuronide. Negative results were obtained by Schmiedeberg and Meyer [1879] with *d*-camphorylglucuronide, by Sera [1914] with orcinol- and vanillin-glucuronides, and by Miwa [1932] with menthylglucuronide. The varying behaviour of emulsin towards conjugated glucuronic acids is explained by Miwa [1932] to be partly due to the activity of the enzyme which seems essentially dependent on method of preparation and degree of ageing. In nature all derivatives of glucose are without exception derivatives of *d*-glucose, and the naturally formed glucuronides also fall into this category. Bornylglucuronide on hydrolysis gives *d*-glucurone and *d*-glucuronic acid. These facts therefore show that the glucuronide present in urine after ingestion of borneol is β-bornyl-*d*-glucuronide. However, there still remains to be considered the optical configuration of the bornyl residue and its influence, if any, on conjugation.

It was found by Magnus-Levy [1907] that when pure *d*- or *l*-borneol was fed separately to dogs or rabbits, pure *d*- or *l*-bornylglucuronide was excreted in the urine and conjugation took place equally well with either isomeride. The conjugated glucuronic acids were isolated as the sodium salts (sodium β-*d*-bornyl-*d*-glucuronide, $[\alpha]_D - 36{\cdot}7°$ in water, sodium β-*l*-bornyl-*d*-glucuronide, $[\alpha]_D - 66{\cdot}7°$

in water). We have fed pure *l*-borneol[1] to dogs and have recovered from the urine pure *β*-*l*-bornyl-*d*-glucuronide, $[\alpha]_{5461} -74{\cdot}6°$ in water. On the other hand, as is shown in Table IV, we find that when a mixture of *d*- and *l*-borneol is fed to dogs preferential conjugation of the *d*-isomeride occurs, and da Cruz [1930], on feeding inactive menthol to dogs found the menthol after liberation from the conjugated derivative to be optically active. In the course of many of our feeding experiments the rotations of the borneol fed, the glucuronide excreted and the borneol recovered from it after hydrolysis were noted (Table IV).

Table IV.

	$[\alpha]_{5461}$	% *d*-isomeride	
Borneol fed	+20° (EtOH)	73·7	
Bornylglucuronide excreted	−47·9 (water)	86·7	Mean increase
Borneol recovered after hydrolysis	+30 (EtOH)	85·5	12·4 %
Borneol fed	+26·5 (EtOH)	81·4	
Bornylglucuronide excreted	−45·6 (water)	93·0	Mean increase
Borneol recovered after hydrolysis	+33 (EtOH)	89·3	9·8 %

	Calculated from figures quoted by Quick [1927]	
	$[\alpha]_D$	% *d*-isomeride
Borneol fed	+5°	56·7
Bornylglucuronide excreted	−48	66; increase 9·3 %
Borneol recovered after hydrolysis	No observation	No observation

Summary.

1. A brief summary including new observations is given of the normal occurrence of glucuronic acid in the urine and blood of various animals.

2. A stereochemical study of the conjugation of *d*- and *l*-borneol with glucuronic acid *in vivo* reveals a preferential conjugation of the *d*-isomeride.

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[1] *l*-Borneol fed had $[\alpha]_{5461} -42{\cdot}7°$ in alcohol; Mitchell [1925] gives the rotations of *d*- and *l*-borneol as $[\alpha]_{5461} \pm 42{\cdot}2°$ in alcohol.

REFERENCES.

Boku and Kin (1931). *J. Chosen Med. Assoc.* **21**, 67.
da Cruz (1930). *Comp. Rend. Soc. Biol.* **105**, 815.
Flückiger (1885). *Z. physiol. Chem.* **9**, 323.
Haas (1876). *Centralbl. med. Wiss.* 149.
Hagemann (1882). *Pflüger's Arch.* **28**, 506.
Hämäläienen (1911). *Skand. Arch. Physiol.* **23**, 297.
Hemingway, Pryde and Williams (1934). *Biochem. J.* **28**, 136.
Hildebrandt (1905). *Hofmeister's Beitr.* **7**, 438.
Hoppe-Seyler (1882). *Z. physiol. Chem.* **7**, 425.
Ishidate (1929). *J. Pharm. Soc. Japan*, **49**, 336.
Kawanishi (1932). *Mitt. med. Akad. Kioto*, **6**, 289.
Lépine and Boulud (1905). *J. Physiol. Path. Gen.* **6**, 775.
—— —— (1906). *J. Physiol. Path. Gen.* **8**, 581.
Letsche (1907). *Z. physiol. Chem.* **53**, 110.

Magnus-Levy (1907). *Biochem. Z.* **2**, 319.
Mayer (1901). *Z. physiol. Chem.* **32**, 518.
—— and Neuberg (1900). *Z. physiol. Chem.* **29**, 256.
Mester (1888). *Z. physiol. Chem.* **12**, 142.
Miwa (1932). *Acta Phytochim.* **6**, 155.
Mitchell (1925). *J. Chem. Soc.* **127**, 208.
Neuberg and Kretschmer (1911). *Biochem. Z.* **36**, 15.
—— and Niemann (1905). *Z. physiol. Chem.* **44**, 97.
—— and Schwenk (1917). *Biochem. Z.* **79**, 387.
Ørskov (1932). *Skand. Arch. Physiol.* **63**, 255.
Pollecoff (1924). *Biochem. J.* **18**, 1252.
Quick (1927). *J. Biol. Chem.* **74**, 331.
Salkowski and Neuberg (1906). *Biochem. Z.* **2**, 308.
Sauer (1930). *Klin. Woch.* **9**, 2350.
Schmiedeberg and Meyer (1879). *Z. physiol. Chem.* **3**, 422.
Sera (1914). *Z. physiol. Chem.* **90**, 258.
Stepp (1919). *Z. physiol. Chem.* **3**, 422.
Tollens (1910). *Z. physiol. Chem.* **67**, 138.
—— and Stern (1910). *Z. physiol. Chem.* **64**, 39.
West, Lange and Peterson (1932). *Biochem. J.* **26**, 1728.

XX. THE BIOCHEMISTRY AND PHYSIOLOGY OF GLUCURONIC ACID.

V. THE SITE AND MECHANISM OF THE FORMATION OF CONJUGATED GLUCURONIC ACID.

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When a substance which is metabolised with difficulty is introduced into the animal body several detoxication mechanisms can come into play, and it is well known that glucuronic acid, glycine, glutamine [Thierfelder and Sherwin, 1915] and sulphuric acid play various rôles in this connection. A scrutiny of the work for the last half century on the metabolism of certain compounds reveals that many of these (*e.g.* certain aliphatic alcohols, phenols, aldehydes, ketones, hydrocarbons, terpenes, aromatic acids, certain amines and heterocyclic nitrogen compounds) can be detoxicated by conjugation with glucuronic acid. This process of conjugation only occurs if the toxic compound carries a hydroxyl group, a $C_6H_5.COOH$, or a $C_6H_5.\overset{|}{C}.COOH$ group, or can form such a group by oxidation (or in some cases by reduction) in the organism. Thus, terpenes are oxidised to the corresponding alcohol and the latter conjugated with glucuronic acid for excretion [Fromm and Hildebrandt, 1901]:

$$C_{10}H_{16} \rightarrow C_{10}H_{15}OH \rightarrow C_{10}H_{15}O.C_6H_9O_6 \text{ (Terpinolglucuronide).}$$

Benzene is oxidised to phenol which is excreted conjugated with glucuronic and sulphuric acids [Braunstein *et al.*, 1931]. Toluene is oxidised to benzoic acid which is excreted conjugated with glycine and glucuronic acid [Epstein and Braunstein, 1931]. Chloral is reduced to trichloroethyl alcohol which is excreted as trichloroethylglucuronic acid [*cf.* Mering, 1882].

In the second stage of the detoxication the toxic body may combine with glucose to form an intermediate glucoside which is then oxidised to the glucuronic acid, or it may combine directly with preformed glucuronic acid. The former possibility was proposed by Fischer and Piloty [1891] as the mechanism by which conjugated glucuronic acids were formed in the animal, but it had then no experimental basis. None the less it has been widely accepted as an established fact [see Armstrong, 1919; Tollens, 1914]. Hildebrandt [1905; 1909] has claimed that on subcutaneous injection of coniferin (coniferylglucoside), syringin (methoxyconiferylglucoside) and bornylglucoside into rabbits the corresponding conjugated glucuronic acids were excreted in the urine. This observation has alternative explanations, either direct oxidation of the glucoside to the conjugated glucuronic acid, or previous hydrolysis of the glucoside in the body to give a free aglucone which combines with preformed glucuronic acid.

These considerations raise two questions: (1) What organ in the body

carries out the detoxication? (2) Is glucoside formation an intermediate stage in the detoxication process? With a view to providing an answer to these two questions it was decided to perfuse, by means of a pump-lung preparation, the isolated kidney alone and in conjunction with other isolated organs, and to investigate the reaction of such perfused organs to glucuronogenic and other substances added to the circulating blood.

Methods.

Four combinations of perfused tissues were used during these investigations and they are briefly described in the order in which they were employed.

(i) *The pump-lung-kidney preparation* (*Preparation A*).

The arrangement for these experiments was essentially the same as those previously described by Hemingway [1931; 1933]. The preparation was made from two dogs. The first, which weighed 16–18 kg., was anaesthetised, and about 500 ml. of blood were taken from the carotid artery. The blood was whipped, twice filtered through fine muslin and, after measurement of the volume, introduced into the apparatus.

A cannula was tied into the pulmonary artery after opening the chest wall and perfusion of the lungs commenced after removal from the thorax.

Meanwhile the second dog was anaesthetised and about 300 ml. of blood were removed. Saline was injected intravenously to replace this blood. The abdomen was opened in the middle line, the left kidney freed from its attachments and the vessels dissected. A cannula was tied in the ureter, the kidney removed and attached to the perfusion apparatus.

(ii) *The pump-lung-kidney-liver preparation* (*Preparation B*).

A liver, perfused by a method analogous to that employed by Bauer *et al.* [1932] was added to the perfused kidney preparation. The arrangement of the apparatus is shown in Fig. 1. From a large reservoir R, blood was taken by two pumps, P_1 and P_2, to supply respectively the renal and hepatic arteries (*R.A.* and *H.A.*) and a constant level reservoir, *P.R.*, which fed blood to the portal vein (*P.V.*). The kidney and the liver were supported on Büchner funnels the bottoms of which were covered with fine muslin filters. The blood which issued from the cut ends of the renal and hepatic veins was eventually gathered into a common funnel, F, again filtered and after perfusing the pulmonary system, collected in the main reservoir. The output of the pump P_1 was adjusted to maintain a pressure of 120–130 mm. Hg in the renal and hepatic arteries, and that of P_2 to keep the portal vein reservoir overflowing. The level of this reservoir was 10–15 cm. above the portal vein.

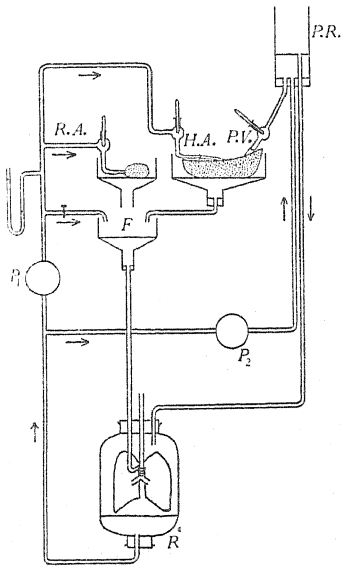

Fig. 1. Arrangement of apparatus in Preparation B.

The main reservoir and the Büchner funnels supporting the organs were placed in thermostats while the tubes conveying blood to and from the main reservoir and the lungs were water-jacketed with water circulated from one of the baths by an "autopulse" pump.

The preparation was made from two dogs and the technique of isolating the organs was identical with that described in the previous section up to the point of removing the kidney. Prior to this, the portal vein was dissected and two loose ligatures placed round it after cutting the splenic and gastric tributaries between ligatures. The hepatic artery was isolated. The aorta was clamped proximal to the renal arteries, the left kidney removed and perfusion commenced.

The portal vein was then cannulated and the liver washed with about 300 ml. of warm saline. The vein and the hepatic artery were clipped and the liver removed. A cannula was tied into the hepatic artery and after filling both cannulae with blood and attaching the appropriate tubes, perfusion was commenced. The first 200 ml. of blood coming from the liver was collected, whipped and filtered before being returned to the blood reservoir.

(iii) *The pump-lung-kidney-spleen preparation* (*Preparation C*).

The spleen vessels were dissected and loose ligatures placed round them. The kidney was removed and perfusion commenced, after which the spleen was removed and a cannula tied in the artery. The organ was then perfused.

(iv) *The pump-lung-kidney-limb preparation* (*Preparation D*).

The hind limb was selected for perfusion. The femoral vessels were dissected and loose ligatures placed round them and round the common iliac artery. The perfusion of the limb was commenced before removing the kidney, so minimising circulatory disturbances in the limb. A cannula was inserted into the femoral artery after tying the common iliac artery. The limb was washed through with defibrinated blood and, after removal by cutting through tissues and bone just below the head of the femur, perfusion was commenced. The blood issuing from the cut end of the femoral vein was collected in a funnel and led to *F*.

In all the experiments, with the exceptions mentioned below, the required amount of the substance whose conjugation was to be tested was dissolved in a small volume of saline and slowly added to the blood passing to the lungs, through funnel *F*.

Experimental.

(*a*) *The site of conjugation of glucuronogenic substances.*

The first organ to be considered was the kidney, since detoxication by conjugation with glycine to form hippuric acids is performed by this organ [Quick, 1932]. It seemed therefore possible that the kidney might perform a like function in regard to glucuronic acid.

As a preliminary it was necessary to demonstrate that the isolated perfused kidney was capable of eliminating preformed conjugated glucuronide. Preparation A was used in this investigation. It should first be stated that normal urine from the dog gives a slight positive test for glucuronides with naphthoresorcinol.

Our tests were carried out as follows: 2 ml. of urine, 2 ml. conc. HCl and 0·5 ml. of naphthoresorcinol (1 % in alcohol) were heated in a large test-tube for 2 mins. in boiling water. The tube was then removed from the water-bath and allowed to stand for 4 mins., and then cooled for 4 mins. in running water. The pigment was extracted with 10 ml. ether. Our experience is that this colour test for glucuronic acid, originally devised by Tollens [1908], although not a strictly quantitative test, is capable of detecting increments of glucuronic acid of the order of 0·25 mg. in 2 ml. urine, and that such increments can be compared colorimetrically.

Urine formed by an isolated kidney, when perfused with normal blood, also gives a positive test. It is, however, much fainter than that of normal urine, and

the intensity of the test falls to an almost zero constant value as the excretion proceeds. The urine from the isolated kidney was collected until it gave this almost zero constant value. At this point blood taken from a dog, which had been previously fed with borneol and which was known to be excreting glucuronide, was added to the circulating blood in the preparation. The result of the experiment is shown graphically in Fig. 2. It will be seen that the addition

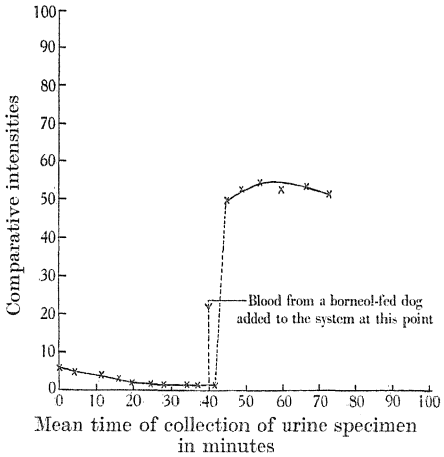

Fig. 2. Graph showing the effect of the addition of blood from a dog fed with borneol, on the intensity of the naphthoresorcinol test performed on urines excreted by an isolated perfused dog kidney. The intensity of the test performed on the bladder urine of the borneol-fed dog is taken as 100 arbitrary units of intensity.

of "borneol-blood" to the circulation of the pump-lung-kidney is followed by an immediate and marked increase of glucuronide in the urine formed by the kidney.

The next step in the investigation was to determine whether or not conjugation occurred in the isolated perfused kidney. The addition of phenol to the perfusing blood (50 mg. in 5 ml. saline to 570 ml. blood) was not followed by any increase in the glucuronide excretion, although the presence of free phenol was readily demonstrated in the urine formed within 5 mins. of the addition of the phenol to the blood. A similar experiment was performed with borneol, but in this case, owing to its low solubility, the crystalline solid was suspended in the circulating blood inside a fine muslin bag. Results as with phenol were negative.

The foregoing experiments showed that preformed conjugated glucuronic acid could readily be eliminated by the isolated perfused kidney, although the negative results in regard to conjugation left the site of formation an open question.

Embden and Glässner [1903] have shown that perfusion of the liver with blood containing phenol results in an increase of glucuronic acid in the perfused blood. A few workers have shown that there is a decreased glucuronic acid output when the liver is injured or non-functional owing to disease [Nazarjanz, 1931; Boku and Kin, 1931]. These experiments suggest that the liver is probably the site of synthesis. The results now to be described prove that this is so and also suggest that the liver alone performs this detoxication process. Preparations B, C and D were used in the series of experiments which lead to this

conclusion. The course of each experiment was similar to that already described in reference to the use of Preparation A. The following substances, all of which are known to form conjugated glucuronides in the intact animal, were added to the perfusion system: phenol, chloral hydrate, camphor and borneol.

(1) *Phenol.* This was added to the blood in two successive lots, (*a*) 30 mg. in 3 ml. normal saline, and (*b*) 70 mg. in 7 ml. normal saline. Within 4 mins. of the addition of each quantity of phenol, using the naphthoresorcinol reaction, marked increases in the glucuronide excretion were observed, (*b*) giving a considerably stronger result than (*a*). 14–15 mins. after the addition of (*b*), the glucuronide elimination by the kidney had sunk almost to zero, so that the process of conjugation and elimination was rapidly completed.

(2) *Chloral hydrate.* In the case of this substance the course of elimination of the conjugated glucuronide is given graphically in Fig. 3, which is constructed

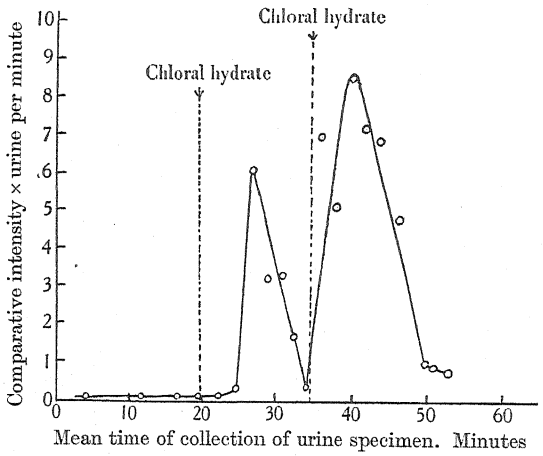

Fig. 3. Graph showing the elimination of glucuronic acid in the pump-lung-kidney-liver preparation in response to added chloral hydrate.

by multiplying the relative intensity (employing arbitrary units) of the naphthoresorcinol test by the rate of urine formation. As before the chloral hydrate was added in two lots, (*a*) 0·19 g. and (*b*) 3·03 g. each in 10 ml. normal saline (to a blood-volume of 1020 ml.) and the subsequent naphthoresorcinol reaction reached a maximum intensity in 5 to 8 mins. after each addition.

It will be observed that the second dose, some 15 times the amount of the first, did not produce a proportional increase in the glucuronide elimination, which no doubt implies some time limitation of the capacity of the liver to conjugate the chloral. It is also clear that, under the conditions of the experiment, only a part of the added chloral hydrate is eliminated *via* the kidney as conjugated glucuronide.

(3) *Camphor.* Camphor was added to the perfusing blood (1070 ml.), (*a*) in 20 ml. of saline which had previously stood in contact with solid camphor for 24 hours at 37°, and (*b*) by placing solid camphor in a fine muslin bag in the circulating system (at *F*, Fig. 1) so that the blood percolated the solid. In both cases marked increases in the glucuronide excreted were observed.

(4) *Borneol.* Solid borneol was percolated by the circulating blood (1015 ml.) as in (3) above. Positive results were again obtained.

In some early experiments the dogs used were initially under chloralose

anaesthesia. In all such cases the urine first formed in the liver-kidney perfusion system gave a marked indication of the presence of conjugated glucuronide. Chloralose, a condensation product of chloral and glucose, contains free chloral which (see (2) above) forms conjugated glucuronide in Preparation B. Chloralose anaesthesia was therefore abandoned after these early experiments.

Although the foregoing experiments proved that the liver is one of the sites of conjugation of glucuronic acid, the possibility remained that other organs might perform a like function. To test this possibility Preparations C and D were devised. The technique used was the same as that employed with Preparation B using phenol as the potential glucuronogenic substance. One experiment was performed with Preparation C, two with Preparation D, and in none of these was any conjugated glucuronide formed.

(*b*) *The mechanism of conjugation of glucuronogenic substances.*

(1) *Experiments with glycosides.* If the glycoside is an intermediate compound in the detoxication process then perfusion of the liver with blood containing glycosides should result in the formation of conjugated glycuronic acids. A second series of experiments was therefore made with Preparation B. In these (1) β-phenyl-*d*-glucoside and (2) β-bornyl-*d*-glucoside were added to the perfusing blood in separate duplicate experiments. The β-isomerides were used since all conjugated glucuronides formed *in vivo* are of the β-glycosidic type. The former glucoside was synthesised by the method of Fischer and Armstrong [1901], the latter by that of Fischer and Raske [1909]. β-Phenyl-*d*-glucoside (105 mg. in 10 ml. saline) was added to the blood (1035 ml.) in the perfusion system, whilst in the case of β-bornyl-*d*-glucoside the crystalline solid (65 mg.) was percolated as before by the blood (775 ml.). At no stage in either experiment after the addition of the glucoside was any increased elimination of glucuronide observed, although the conditions employed were identical with those giving positive results with known glucuronogenic substances. It should be added that in the case of β-phenyl-*d*-glucoside, the unchanged substance was found in the urine within 5 mins. of its addition to the perfusing blood. No free phenol was detected.

Another experiment was performed in which β-bornyl-*d*-galactoside (synthesised by the method of Robertson [1929]) was added to Preparation B. No evidence of the formation of conjugated galacturonic acid was obtained.

(2) *The influence of cyanide on the conjugation process.* The process of conjugation may require the cellular integrity of the liver or, on the other hand, it may depend on the existence of a preformed enzyme. In the former event one might expect the process of conjugation to be inhibited or abolished by cyanide. A suitable experiment, performed with Preparation B in which KCN (0·003 *M*) was added to the perfusing blood, confirmed this expectation, since added phenol was eliminated in the urine unchanged and no glucuronic acid was formed.

SUMMARY.

1. A description is given of a new technique for perfusing various organs (liver, spleen, tissues of the hind limb) in combination with the kidney.

2. It is demonstrated by using such preparations that the main, if not the only, site of formation and conjugation of glucuronic acid is the liver. The process of conjugation is abolished by cyanide.

3. No support is found for the hypothesis that the production of a glucoside is an intermediate stage in glucuronide formation.

The cost of this investigation was defrayed in part by grants from the Royal Society and from the Medical Research Council. One of us (R. T. W.) is indebted to the Medical Research Council for a whole time assistance grant.

REFERENCES.

Armstrong (1919). The simple carbohydrates and the glucosides, 3rd edition, p. 56. (London: Longmans, Green and Co.)
Bauer, Dale, Poulsson and Richards (1932). *J. Physiol.* **74**, 343.
Boku and Kin (1931). *J. Chosen Med. Assoc.* **21**, 67.
Braunstein, Parschin and Chalisowa (1931). *Biochem. Z.* **235**, 311.
Embden and Glässner (1903). *Centr. Physiol.* **16**, 778.
Epstein and Braunstein (1931). *Biochem. Z.* **235**, 328.
Fischer and Armstrong (1901). *Ber. deutsch. chem. Ges.* **34**, 2885.
—— and Piloty (1891). *Ber. deutsch. chem. Ges.* **24**, 521.
—— and Raske (1909). *Ber. deutsch. chem. Ges.* **42**, 1465.
Fromm and Hildebrandt (1901). *Z. physiol. Chem.* **33**, 574.
Hemingway (1931). *J. Physiol.* **71**, 201.
—— (1933). *J. Physiol.* **77**, 14 P.
Hildebrandt (1905). *Beitr. chem. Physiol. Pathol.* **7**, 438.
—— (1909). *Biochem. Z.* **21**, 1.
Mering (1882). *Z. physiol. Chem.* **6**, 480.
Nazarjanz (1931). *Z. exp. Med.* **30**, 11.
Quick (1932). *J. Biol. Chem.* **96**, 73.
Robertson (1929). *J. Chem. Soc.* 1820.
Thierfelder and Sherwin (1915). *Z. physiol. Chem.* **94**, 1.
Tollens (1908). *Ber. deutsch. chem. Ges.* **41**, 1788.
—— (1914). Handbuch der Kohlenhydrate, 3rd edition, p. 762. (Leipzig.)

XXI. GLYCEROPHOSPHORIC ACID.

I. THE RECOGNITION AND SEPARATION OF α- AND β-GLYCEROPHOSPHATES.

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Synthetic glycerophosphates have been known for many years and have been prepared in several ways. The acid may exist in two isomeric forms, α and β. Carré [1903] showed that glycerol when esterified with phosphoric acid at 105° yielded almost entirely monoglycerylphosphoric acid. At higher temperatures further condensation took place. Later Power and Tutin [1905] prepared synthetic glycerylphosphates by a similar method and also obtained the mono-derivatives. Poulenc Frères [1907] showed that a well-crystallised sodium glycerylphosphate could be prepared by heating monosodium phosphate with two molecular proportions of glycerol and hydrolysing the resulting di-ester with sodium hydroxide. All these methods yielded mixtures of α- and β-glycerophosphates.

There are three principal methods for preparing pure α- and β-glycerophosphates. (1) King and Pyman [1914] mixed pure α-monochlorohydrin and trisodium phosphate, and on working up the product obtained pure α-glycerophosphate. Pure β-glycerophosphate was obtained from α-dichlorohydrin and phosphoryl chloride. (2) Bailly [1915] prepared pure α-glycerophosphate from epichlorohydrin. (3) Fischer and Pfähler [1920] prepared pure α-glycerophosphate from acetone-glycerol, phosphorus oxychloride and quinoline.

The following methods have been advanced to distinguish between the α- and β-isomerides.

1. Grimbert and Bailly [1915] found that the α-ester on careful oxidation (so as to avoid hydrolysis) yields a compound containing a ketone group, which can be identified by the Denigès reaction. The β-form on the other hand does not give this reaction.

2. Karrer and Salomon [1926] described a method which depends on the fact that barium β-glycerophosphate forms with barium nitrate a difficultly soluble double salt which precipitates on standing. The α-form does not give this double salt. Kay [1931] has criticised Karrer and Salomon's results on the ground that they used supersaturated solutions from which a salt would crystallise out on standing without the addition of barium nitrate. We have, however, put this method to an important use to be described later.

3. Since Levene and Yawagama [1920] had found that the rate of hydrolysis of the phosphoric esters of the sugars with sulphuric acid varied with the position of the phosphate group, it was thought advisable to see if the α- and β-forms of

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glycerophosphate would show this peculiarity. To test this point solutions of the α- and β-barium glycerophosphates were hydrolysed with N sulphuric acid at 100°. k was calculated for a unimolecular reaction. The average values for one experiment were for the α, $k=0{\cdot}00281$, and for the β, $k=0{\cdot}00266$. From these results it is evident that the rates of hydrolysis of the two isomerides with strong acids are not appreciably different.

4. *The enzymic hydrolysis method* for distinguishing between the two isomerides depends upon the fact that both animal and plant phosphatases hydrolyse the two forms at markedly different rates. Kay [1926] has shown that mammalian kidney phosphatase attacks the β- more rapidly than the α-isomeride. Earlier workers had stated that the α- and β-forms were attacked at the same rate. A plant phosphatase was investigated by a group of Japanese workers [Akamatsu, 1923; Inouye, 1928; Kobayashi, 1927; Asakawa, 1929], who showed its presence in takadiastase. It has been found by Kay and Lee [1931] that with most mammalian phosphatases and with takaphosphatase the β- is attacked more rapidly than the α-form. This fact has been made the basis for their method of distinguishing between the two isomerides. With phosphatase from *Propionibacterium jensenii* [Werkman and Kendall, 1931] and with blood-phosphatase, as shown by Roche [1931], the α- is attacked more rapidly than the β-form.

In the present investigation a study has been made of the rates of enzymic hydrolysis of pure β- and of a specially purified α-glycerophosphate. Purification of the α- from small amounts of the β-isomeride has been accomplished both by the use of Karrer's double salt method and by partial enzymic hydrolysis of the impure material. Since the β-isomeride is hydrolysed more rapidly than the α-form, the former is removed and although there is some loss of the α-form, it is obtained pure towards the end of the reaction.

In the expectation that the α-glycerophosphate might consist of two optical enantiomorphs with different rates of enzymic hydrolysis, an attempt was made to effect a separation of the one enantiomorph from the other by means of the partial enzymic hydrolysis of purified α-glycerophosphate. An attempt was also made to resolve the α-form by means of fractional crystallisation of several of its alkaloidal salts. None of the fractions obtained by the method of partial hydrolysis or by fractional crystallisation appeared to have any significant degree of optical rotation. In a second communication [Rae, 1934], the results are presented of a study of the relative amounts of the α- and β-isomerides in the glycerophosphates derived from the naturally occurring phosphatides.

Experimental.

Materials. The crystalline salt of sodium β-glycerophosphate, obtained from Boot's Pure Drug Company, appeared to consist almost entirely of the β-isomeride. It gave only a very slight Denigès reaction, and no sample of β-glycerophosphate has been obtained or prepared in this laboratory which has a higher rate of enzymic hydrolysis. On the other hand, several samples of the α-isomeride, which have been obtained from time to time, have appeared to contain different and widely variable amounts of the β-isomeride. Because of the discrepancies in the various experiments in which the reputedly pure α-compound was used, it was thought advisable to prepare some of the material from authentic α-monochlorohydrin by King and Pyman's [1914] method. For this purpose Eastman Kodak monochlorohydrin was purified by fractional distillation under reduced pressure until a fraction of constant boiling-point was obtained. 50 g. of this material were condensed with phosphoric acid, and 49 g. of calcium α-glycerophosphate were obtained, 52 % of the theoretical yield.

The results of several experiments with reputed α-glycerophosphate were communicated to Dr F. L. Pyman, of Boot's Pure Drug Company, who very kindly prepared a special batch of the α-salt, which appeared practically identical with that prepared in Toronto.

Methods of estimating rates of enzymic hydrolysis.

(1) *By animal phosphatase.*

Enzyme. Phosphatase occurs abundantly in liver, kidney, intestine and bone. For extraction the fresh tissue is ground up with 10–20 times its weight of chloroform water and allowed to stand for 48 hours at room temperature. It is then filtered through cotton-wool or coarse filter-paper and is ready for use.

Substrates. Approximately *M*/100 aqueous solutions of sodium glycerophosphate were used. These were prepared from the barium salts by adding an equivalent amount of sodium sulphate. The various substrates to be compared were analysed for phosphorus colorimetrically [King, 1932] and diluted to the same concentration.

Buffers. The optimum p_H for mammalian phosphatase acting on glycerophosphates is between 8·9 and 9·2. The following buffers were used in different experiments.

(i) Sørensen's glycine buffer, p_H range 8·53–12·90 [Clark, 1928].
(ii) Palitzsch's borate buffer, p_H range 6·77–9·24 [Clark, 1928].
(iii) Michaelis's veronal buffer, p_H range 6·8–9·6 [Michaelis, 1930].

Method. The hydrolyses are carried out as follows. A series of tubes at a range of p_H covering the optimum is prepared (4 for each p_H), using for each 5 ml. substrate (adjusted to the desired p_H), 5 ml. buffer and 1 ml. of the enzyme solution. To two of each set of 4 tubes (the controls) are immediately added 2 ml. of 25 % trichloroacetic acid; after 10 minutes the contents are filtered. The other 2 tubes are placed in a water-bath at 37·5° for exactly 2 hours. These are then removed, 2 ml. of 25 % trichloroacetic acid added and the contents filtered after

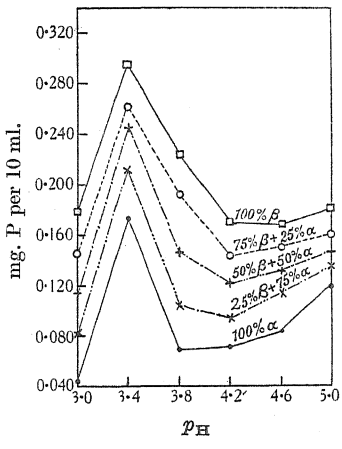

Fig. 1.

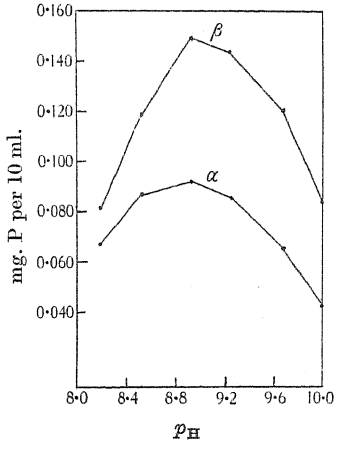

Fig. 2.

Fig. 1. Rates of hydrolysis by takaphosphatase of pure α-; 75 % α-, 25 % β-; 50 % α-, 50 % β; 25 % α-, 75 % β-; and pure sodium β-glycerophosphates. Phthalate buffers used.

Fig. 2. Rates of hydrolysis by mammalian phosphatase of pure α- and β-glycerophosphates. Glycine buffers used.

10 minutes. 10 ml. of filtrate from each tube are then analysed for inorganic phosphorus by a colorimetric method. The increase in inorganic phosphorus, expressed in mg. P per 10 ml. of reaction mixture, represents the amount of hydrolysis. In no case was the amount of hydrolysis a large fraction of the total substrate present.

(2) *By plant phosphatase.*

Enzyme. 0·5 ml. of 2 % takadiastase solution freshly prepared.

Buffers. The optimum p_H for plant phosphatase is 3·0–5·0.

(i) Clark and Lub's phthalate buffer, p_H range 2·2–6·2 [Clark, 1928].

(ii) Michaelis's [1931] acetate-veronal buffer, p_H range 2·62–9·64.

Substrates. These were the same as with mammalian phosphatase except that the p_H was adjusted to 4·2 before adding the buffer or the enzyme. This was the mean p_H of the several buffer mixtures used, and the final p_H of the mixtures did not differ appreciably from that of the buffer added in any case. The other details were the same as for the mammalian phosphatase.

Figs. 1 and 2 show the type of results obtained with pure α- and β-glycerophosphates using mammalian phosphatase and takaphosphatase.

Separation of the α-isomeride from the β-isomeride by enzymic means.

If pure α- or β-glycerophosphate is hydrolysed enzymically and fractions are removed from the reaction mixture as the hydrolysis proceeds, these fractions should show no difference in their rates of enzymic hydrolysis; but if a mixture of α- and β-glycerophosphates be used there should be a progressive change in the rates of hydrolysis of the fractions removed, since the β-form is hydrolysed faster than the α-isomeride. A large amount of sodium glycerophosphate was hydrolysed by the enzyme and portions of the reaction mixture were removed at the start and at approximately 25, 50 and 75 % hydrolysis. These fractions were freed from inorganic phosphorus by adding the calculated amount of magnesia

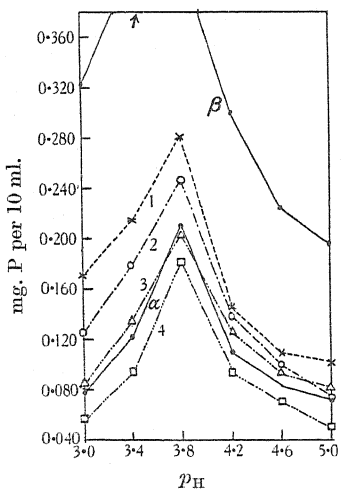

Fig. 3.

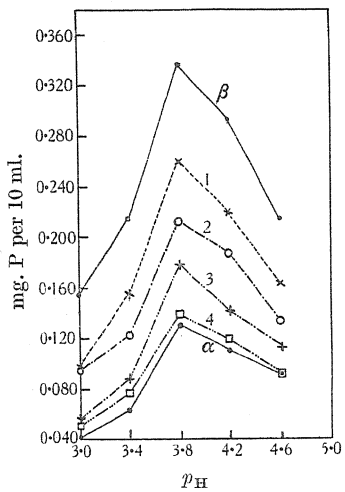

Fig. 4.

Fig. 3. Rates of hydrolysis by takaphosphatase of fractions (1, 2, 3, 4) obtained by hydrolysing reputed sodium α-glycerophosphate (source 1) with takaphosphatase.

Fig. 4. Rates of hydrolysis by takaphosphatase of fractions (1, 2, 3, 4) obtained by hydrolysing reputed sodium α-glycerophosphate (source 2) with takaphosphatase.

mixture without the ammonia. The solutions were then made pink to phenolphthalein by adding ammonia and the inorganic phosphate precipitated was centrifuged off. To the centrifugate was added an excess of normal lead acetate which precipitated the lead glycerophosphate. This was decomposed with hydrogen sulphide, and the solution, freed from this reagent, was made alkaline to phenolphthalein with baryta.

The barium glycerophosphate was precipitated by adding the filtrate to four volumes of alcohol. When two samples of reputed α-glycerophosphate, obtained from two different sources, were treated in this way and the several fractions compared for rates of enzymic hydrolysis, the results shown in Figs. 3 and 4 were obtained. These findings indicate that the α-salt as supplied to us contained some of the β-isomeride as an impurity.

Separation by double salt precipitation with barium nitrate.

An attempt was therefore made to purify commercial calcium α-glycerophosphate, after conversion into the barium salt, by a modification of Karrer and Salomon's [1926] method for differentiating the α- from the β-form. 30 g. of the barium salt dissolved in 300 ml. of water and 24 g. of barium nitrate dissolved in 300 ml.of water were mixed and allowed to stand in the refrigerator. The solution was reduced in volume until a precipitate had formed. After filtering, the filtrate was added to alcohol and the precipitated barium glycerophosphate was dissolved

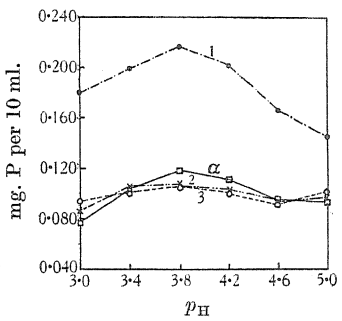

Fig. 5. Rates of hydrolysis by takaphosphatase of reputed sodium α-glycerophosphate before and after treatment with barium nitrate; (1) before treatment, (2) one treatment, (3) two treatments. α- refers to authentic α-acid.

in water and reprecipitated. This process was repeated to remove all the barium nitrate. The salt was again treated with the required amount of barium nitrate, and after standing, the barium glycerophosphate was again separated. The original salt and the salt after one, and after two, treatments were then compared as to their rates of enzymic hydrolysis. The results are given in Fig. 5. Judging by the rates of enzymic hydrolysis, one treatment with barium nitrate is sufficient to free the α-salt from the contaminating β-salt.

Attempts to separate α-glycerophosphate into optical enantiomorphs.

The method used above for estimating the relative amounts of α- and β-isomerides in a mixture of the two is open to a serious objection. Since the α-form has an asymmetric carbon atom in its formula, it might be expected that *d*-, *l*-, racemic and *dl*-, forms might all exist, and that these various forms might be hydrolysed at different rates. If this were so, what is considered from

the graph to be a mixture of the α- and β-isomerides might in fact contain no β-form but might be only the *d*- or *l*- form of the α-salt being hydrolysed at a faster rate than the authentic racemic α-salt. To settle this problem an attempt was made to resolve the synthetic α-form into its optical antipodes. Some earlier workers have suggested that the naturally occurring α-form was optically active. Levene and Rolf [1919] found slight activity in glycerophosphates from natural sources. The amount of rotation depended to a large extent on the method and the amount of purification used. Karrer and Benz [1926, 1, 2; 1927], after carefully separating the α- and β-forms from natural sources, obtained no rotation for either salt. They consider that earlier workers who found activity were probably observing the rotation of some impurity. Karrer and Benz, although they failed to obtain a separation with either strychnine or quinine, still believe that the α-form is optically active, because they prepared a dimethyl ether of the dimethyl ester of the α-salt which showed some slight activity. Abderhalden and Eichwald [1918] endeavoured to prepare the α-salt in its active form by acting on *d*-monobromohydrin dissolved in pyridine with phosphorus oxychloride in the cold. After a lengthy procedure, they separated the glycerophosphoric acid as the lithium salt. The analysis gave values too low in phosphorus and too high in carbon. The observed rotation was +0·48°, giving a specific rotation of +6·26°. In none of these attempts has conclusive evidence been given for the resolution of the α-salt.

The following attempts have been made to resolve the α-salt into its optical enantiomorphs.

(*a*) *By enzymic means.* Takaphosphatase was the enzyme used. The method was similar to that described above when the purification of the commercial α-salt was discussed. The three or four fractions obtained were analysed for phosphorus and barium, and their optical activity (Hg green) and their rates of enzymic hydrolysis were compared, using both mammalian and takaphosphatase. The results obtained when purified α-glycerophosphate or the synthetic product

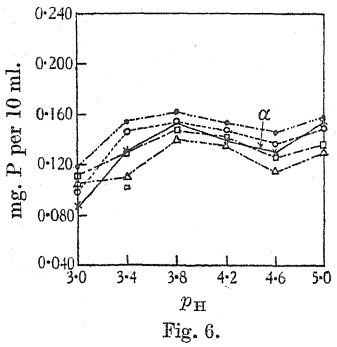

Fig. 6.

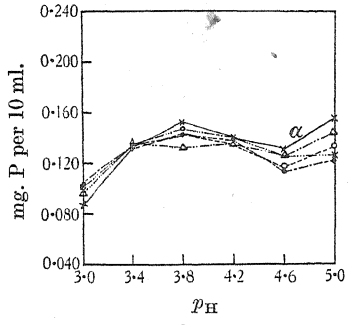

Fig. 7.

Fig. 6. Rates of hydrolysis by takaphosphatase of fractions obtained by hydrolysing with takaphosphatase sodium α-glycerophosphate purified by means of barium nitrate. The solid line is given by an authentic α-salt.

Fig. 7. Rates of hydrolysis by takaphosphatase of fractions obtained by hydrolysing with takaphosphatase sodium α-glycerophosphate prepared synthetically by the Pyman method. The solid line is given by an authentic α-salt.

was used are shown in Figs. 6 and 7. These graphs indicate that if any resolution has occurred, it is without effect on the rates of enzymic hydrolysis. The optical rotations were all less than the experimental error.

Propionibacterium jensenii contains a phosphatase which hydrolyses glycerophosphates at an optimum p_H of 7·0. To test the effect of this enzyme a large amount of these bacteria was obtained. By following a similar treatment to that described above for takaphosphatase, fractions were obtained at various percentages of hydrolysis, and these were compared as to their optical activity and enzymic activity. There was no significant difference between the fractions.

(*b*) *By the use of active alkaloids.* Brucine glycerophosphate was prepared by treating 10 g. of Ca α-glycerophosphate with sufficient *N* oxalic acid to form the free acid. The calcium oxalate was centrifuged off, and to the filtrate was added a slight excess of brucine dissolved in 50 % alcohol. The fractions which crystallised out were dissolved in 20 % alcohol, and a slight excess of barium acetate was added. The mixture was poured slowly into four volumes of alcohol and the barium glycerophosphate formed was filtered off. This salt was reprecipitated for purification. The fractions were analysed for phosphorus and compared as to rates of enzymic hydrolysis and optical activity. The results showed that, although there was a slight optical rotation in all fractions, there was no definite shift in values from one member of the series to the next. The results were with one or two exceptions within the experimental error. The resolution, if any, was without effect on the rates of enzymic hydrolysis.

A second experiment was done without converting the fractions into the barium salts. In this there was a slight shift in rotation, but since brucine itself has such a high negative rotation this might be due to varying traces of brucine.

Quinine glycerophosphate was made by mixing quinine sulphate with barium glycerophosphate. After filtering off the barium sulphate formed the mixture was allowed to crystallise spontaneously. The results from these fractions indicated that the optical rotations were not of sufficient magnitude to be considered significant. Quinine glycerophosphate was then prepared using the free alkaloid instead of the sulphate. Table I gives the results obtained.

Table I.

Fraction	Quinine glycero-phosphate g.	P %	$[\alpha]_D$	Enzyme experiments mg. P hydrolysed	
				Mammalian phosphatase	Taka-phosphatase
1	10·00	4·40	−0·4°	0·238	0·129
2	14·00	3·36	−0·1	0·236	0·142
3	16·00	3·42	−0·1	0·245	0·148
4	9·00	3·45	+0·1	0·269	0·145
5	11·00	3·30	0·0	0·261	0·142
6	7·05	3·44	0·0	0·241	—
7	7·96	3·35	−0·4	0·252	—
8	7·00	3·30	−0·2	0·229	0·132

These results do not show any decided drift of optical activity in either direction. Another experiment was done without conversion into the barium salt. The fractions obtained were analysed for nitrogen and phosphorus and their solubilities determined. There was no significant difference in solubility.

From these four attempts it was concluded that there had been insufficient evidence to indicate any resolution of the α-glycerophosphate into its optical antipodes by means of the active alkaloids quinine and brucine.

There remained the possibility that a resolution had occurred but that the optical rotations of the fractions obtained were too small to measure. An attempt was therefore made to obtain an increase in rotation by the addition of tungstates

and molybdates as Gernez [1887; 1888; 1889, 1, 2] had shown to be the case with tartaric acid. When a molecular proportion of sodium molybdate was added to dilute solutions of tartaric acid and α-glycerophosphoric acid the following results were obtained.

Table II.

Salt used	g. dissolved in 15 ml.	Observed rotation before	Observed rotation after	Increase in rotation
d-Tartaric acid	0·150	+0·4°	+13·33°	33·3 times
α-Glycerophosphoric acid	0·172	0	0	0

These results were obtained with Dr Pyman's authentic synthetic α-salt and two fractions from enzymic hydrolysis of the synthetic α-salt. A similar result was obtained using sodium tungstate in place of sodium molybdate. When two molecular proportions of either sodium molybdate or tungstate were used the result was the same. Apparently the addition of these salts to the glycerophosphates does not enhance the rotation, if there is such, to a point where it is detectable.

Summary.

Various methods for determining the relative amounts of α- and β-glycerophosphates are described, and the application of one of these to unknown glycerophosphates is given in detail.

An enzymic method for detecting small amounts of the β-isomeride in reputed α-salts is described and a method outlined for the purification of the α-salt.

An attempt has been made to resolve the α-salt into its optical isomerides using an enzymic method and the alkaloids brucine and quinine. No positive evidence of separation has been obtained.

The addition of molybdates and tungstates does not appear to increase the rotation of glycerophosphoric acid to a point where it can be read.

REFERENCES.

Abderhalden and Eichwald (1918). *Ber. deutsch. chem. Ges.* **51**, 1308.
Akamatsu (1923). *Biochem. Z.* **142**, 184.
Asakawa (1929). *J. Biochem. (Japan)*, **11**, 143.
Bailly (1915). *Compt. Rend. Acad. Sci.* **160**, 663.
Carré (1903). *Compt. Rend. Acad. Sci.* **137**, 1070.
Clark (1928). The determination of hydrogen ions. (The Williams and Watkins Company, Baltimore.)
Fischer and Pfähler (1920). *Ber. deutsch. chem. Ges.* **53**, 1606.
Gernez (1887). *Compt. Rend. Acad. Sci.* **104**, 784.
—— (1888). *Compt. Rend. Acad. Sci.* **106**, 1527.
—— (1889, 1). *Compt. Rend. Acad. Sci.* **108**, 942.
—— (1889, 2). *Compt. Rend. Acad. Sci.* **109**, 769.
Grimbert and Bailly (1915). *Compt. Rend. Acad. Sci.* **160**, 207.
Inouye (1928). *J. Biochem. (Japan)*, **10**, 133.
Karrer and Benz (1926, 1). *Helv. Chim. Acta*, **9**, 23.
—— —— (1926, 2). *Helv. Chim. Acta*, **9**, 598.
—— —— (1927). *Helv. Chim. Acta*, **10**, 87.
—— and Salomon (1926). *Helv. Chim. Acta*, **9**, 3.
Kay (1926). *Biochem. J.* **20**, 791.
—— (1931). *J. Biol. Chem.* **93**, 409.

Kay and Lee (1931). *J. Biol. Chem.* **91**, 135.
King, E. J. (1932). *Biochem. J.* **26**, 292.
King, H. and Pyman (1914). *J. Chem. Soc.* **105**, 1238.
Kobayashi (1927). *J. Biochem.* (*Japan*), **8**, 205.
Levene and Rolf (1919). *J. Biol. Chem.* **40**, 1.
—— and Yawagama (1920). *J. Biol. Chem.* **43**, 323.
Michaelis (1930). *J. Biol. Chem.* **87**, 33.
—— (1931). *Biochem. Z.* **234**, 139.
Poulenc Frères (1907). *F.P.* 373,112.
Power and Tutin (1905). *J. Chem. Soc.* **87**, 249.
Rae (1934). *Biochem. J.* **28**, 152.
Roche (1931). *Biochem. J.* **25**, 1724.
Werkman and Kendall (1931). *Iowa State College J. of Sci.* **6**, 17.

XXII. GLYCEROPHOSPHORIC ACID.

II. THE GLYCEROPHOSPHORIC ACID OF THE NATURALLY OCCURRING PHOSPHATIDES.

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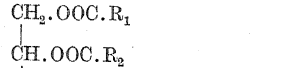
From the Department of Medical Research of the University of Toronto.

(*Received November 14th, 1933.*)

The naturally occurring phosphatides are usually represented as compounds of fatty acids and a base with the α- (unsymmetrical) variety of glycerophosphate (*e.g.* lecithin I), but the possibility has long been recognised that the β-variety (lecithin II) may also occur.



I

II

Glycerophosphoric acid can be obtained by hydrolysing the phosphatides with baryta. From this acid, as it occurs in egg-lecithin, liver-lecithin, brain-lecithin, kephalin and calcium phosphatidate, barium salts were obtained and these were investigated as to the relative amounts of the α- and β-isomerides present, using an enzymic method. Some objections as to the applicability of this method were removed.

Willstätter and Lüdecke [1904] found that the glycerophosphoric acid prepared from egg-lecithin was optically active, and they therefore concluded that it was the α-form. This observation may prove the presence of the α-form but does not exclude the possibility of the β-isomeride being present. Tutin and Hahn [1906] maintained that both the α- and β-forms were present. Grimbert and Bailly [1915] introduced a simple chemical test for differentiating the two isomerides which was based on the Denigès reaction, and using this test they showed that lecithin was a mixture of both varieties. Levene and Rolf [1919], using a highly purified egg-lecithin, obtained a glycerophosphate which had an optical rotation of $-0{\cdot}74°$. Willstätter and Ludecke's value was $-1{\cdot}71°$. Both groups of workers assert that the magnitude of the rotation depends very much on the mode of preparation.

Karrer and Salomon [1926] have shown, using a double salt precipitation method, that both varieties occur in lecithin. Owing to the confusion in this field and to the fact that Kay and Lee [1931] had recently brought forward a quantitative method for estimating the relative amounts of the α- and β-isomerides present in a mixture, it was thought advisable to prepare several phosphatides and estimate the relative amounts of the two isomerides in the glycerophosphates prepared from them.

[1] An abstract from a thesis submitted in conformity with the requirements for the Degree of Doctor of Philosophy in the University of Toronto.

Experimental.

Preparation of the phosphatides.

Lecithin was prepared from liver by a modification of Levene's [1921] method. 43 pounds of fresh ox-liver were minced and dried *in vacuo* at 37°. The material was finely ground and extracted with large volumes of alcohol. The extract was reduced in volume and allowed to stand overnight at 0°. This precipitated the cerebrosides. To the filtrate was added a saturated solution of cadmium chloride in hot ethyl alcohol until precipitation was complete. The crude lecithin cadmium chloride was repeatedly washed with ether to remove the kephalin. The purified salt was suspended in chloroform and decomposed with ammonia gas. After distilling off the chloroform the residue was dissolved in a minimum volume of ether and precipitated by adding it to a large volume of acetone. The yield was 69·8 g. Commercial egg-lecithin was purified by treating the material with cadmium chloride and recovering the lecithin in the same manner as that described above.

Brain-lecithin and brain-kephalin were prepared in the following way. 60 pounds of fresh ox-brain were freed from adhering tissue, minced and extracted with large volumes of acetone to dry the material and to free it from neutral fat. The brain was extracted with ether and the extract reduced to a small volume and allowed to stand at 0° for 15 hours. The supernatant liquid, now free from cerebrosides, was poured into alcohol. The alcohol-soluble part contained the lecithin. This was separated from the solution in a manner analogous to that described in the preparation of liver-lecithin. The kephalin was dissolved in ether and again allowed to stand at 0°, during which time more cerebrosides separated out. This process was repeated until no further precipitation occurred on standing. The liquid was added to alcohol and the kephalin precipitated. The yield was 455 g.

Chibnall and Channon [1927, 1, 2, 3] showed that the ether-soluble substances of cabbage-leaf cytoplasm contain little, if any, phospholipin in the commonly accepted use of the term, the major part of the lipin being present as the calcium salt of a diglyceridephosphoric acid. The calcium salt of phosphatidic acid was prepared in the following way. Four pounds of dried cabbage were exhaustively extracted with ether in a modified Soxhlet apparatus. The extract was evaporated to dryness, taken up in a small volume of ether and precipitated by adding to four volumes of acetone. The part insoluble in hot acetone gave the following results on analysis: P 4·00, 3·92 %; Ca 5·50, 5·45 %; Ca/P = 1·07. Calculated for Ca distearylglycerophosphoric acid: P 4·18 %; Ca 5·39 %; Ca/P = 1.

Preparation of barium glycerophosphate from the phosphatides.

The phosphatide was dissolved in a suitable solvent and emulsified by adding it slowly to an equal volume of hot water. This emulsion was then added to a hot baryta solution and boiled for one hour on the water-bath. The fatty acid formed barium soaps, and these were filtered off and washed with hot water. The filtrate and washings were treated with carbon dioxide to remove excess barium. The filtrate was reduced in volume and a slight excess of normal lead acetate was added. The lead glycerophosphate was washed, suspended in water and decomposed with hydrogen sulphide. The solution was freed from hydrogen sulphide and made neutral to phenolphthalein by the addition of baryta. It was then added to four volumes of alcohol, and the precipitated barium glycerophosphate was washed with alcohol and ether and dried *in vacuo* over sulphuric acid. In this

manner barium glycerophosphates were prepared from egg-, liver-, and brain-lecithin, brain-kephalin and calcium phosphatidate.

These salts were analysed for barium, using a micro-gravimetric method. This method makes use of the filter stick (Stäbchen) and the residue is weighed as barium sulphate. Another method used was the titrimetric benzidine method described by King [1932, 1]. The phosphorus was estimated colorimetrically, using King's [1932, 2] method.

Estimation of relative amounts of α- and β-isomerides in glycerophosphates from natural sources.

Both animal and plant phosphatases hydrolyse α- and β-glycerophosphates at different rates. It was decided, therefore, using Kay and Lee's [1931] enzymic method, to determine the relative amounts of the α- and β-isomerides of glycerophosphoric acid occurring in the natural phosphatides. Solutions of authentic

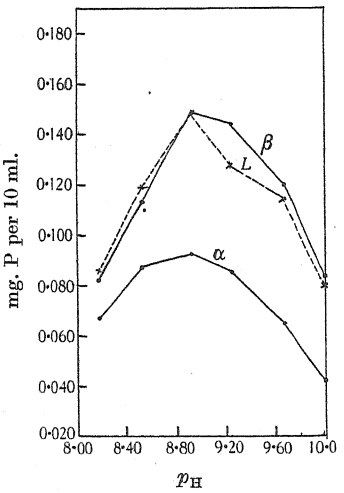


Fig. 1.

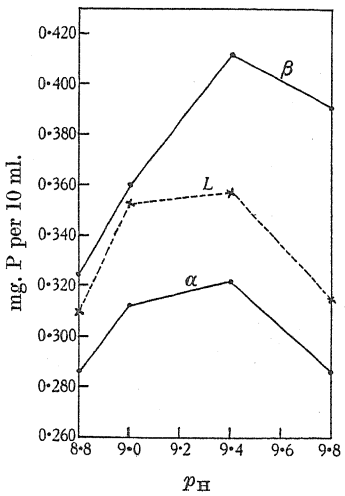


Fig. 2.

Fig. 1. Rates of hydrolysis by mammalian phosphatase of pure α- and β-glycerophosphates and of the glycerophosphate from egg-lecithin. Glycine buffer used.

Fig. 2. Rates of hydrolysis by mammalian phosphatase of pure α- and β-glycerophosphates and of the glycerophosphate from liver-lecithin. Veronal buffer used.

α- and β-salts, and of the unknown glycerophosphate were made up approximately $M/100$, analysed for phosphorus and diluted to exactly the same strength. Rates of hydrolysis were measured by the method described in the previous paper [Rae *et al.*, 1934]. From these curves it may be concluded that the glycerophosphate from egg-lecithin (Fig. 1) is a mixture of α- and β-forms in which the β-form predominates. Liver-lecithin (Figs. 2 and 6) is a mixture of about equal proportions of α- and β-forms. Brain-lecithin (Fig. 3), brain-kephalin (Fig. 4), and calcium phosphatidate (Fig. 5) contain predominantly the α-form. The study of rates of enzymic hydrolysis thus furnishes a simple method for determining the relative amounts of the α- and β-isomerides occurring in the natural phosphatides.

The objection that since the α-form contains an asymmetric carbon, what is considered a mixture of α- and β-forms may be only one of the optical isomerides

of the α-form, has been dealt with in a previous paper [Rae *et al.*, 1934], where it has been shown that in several attempts to resolve the α-form, in no case were fractions obtained that exhibited any difference in their rates of enzymic hydrolysis.

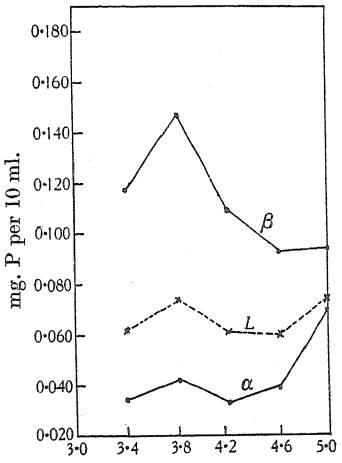


Fig. 3.

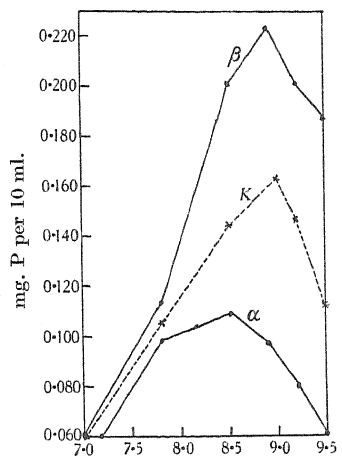


Fig. 4.

Fig. 3. Rates of hydrolysis by takaphosphatase of pure α- and β-glycerophosphates and of the glycerophosphate from brain-lecithin. Phthalate buffer used.

Fig. 4. Rates of hydrolysis by mammalian phosphatase of pure α- and β-glycerophosphates and of the glycerophosphate from brain-kephalin. Glycine buffer used.

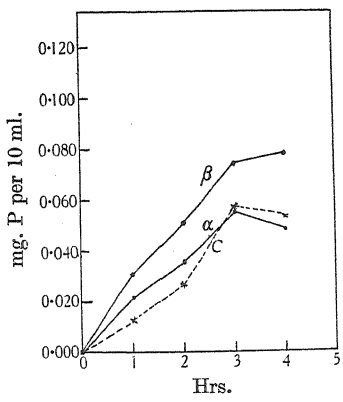


Fig. 5.

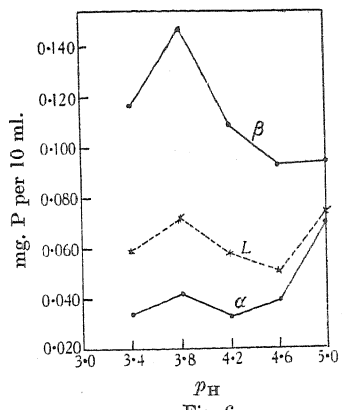


Fig. 6.

Fig. 5. Rates of hydrolysis by mammalian phosphatase of pure α- and β-glycerophosphates and of the glycerophosphate from calcium diglyceridephosphoric acid from cabbage. All at p_H 8·9.

Fig. 6. Rates of hydrolysis by takaphosphatase of pure α- and β-glycerophosphates and of the glycerophosphate from liver-lecithin. Phthalate buffer used.

Another objection is that a wandering of the phosphate group from the α- to the β-position or *vice versa* may occur during the hydrolysis of the phosphatide with baryta. Thus a true picture of the relative amounts of the two isomerides present would not be obtained. To test this, samples of both α- and β-glycero-

phosphates were boiled for 2 hours with the customary amount of baryta. These salts were then compared enzymically and no change in the characteristic rate of hydrolysis was observed in either of them.

These results clearly indicate that the glycerophosphoric acid of the natural phosphatides consists of both the α- and β-isomerides. The formulae of these phosphatides should be written to represent the probability of the occurrence of the symmetrical β-variety, and not always as the asymmetrical α-type.

Summary.

The relative amounts of α- and β-glycerophosphates occurring in several natural phosphatides have been determined by an enzymic method. Egg-lecithin contains a glycerophosphoric acid which is predominantly the β-form; liver-lecithin contains about equal amounts of the α- and β-forms; whereas brain-lecithin, brain-kephalin and calcium phosphatidate contain predominantly the α-form.

My thanks are due to Dr E. J. King, who directed this research, and to Dr H. D. Kay, under whose direction some of the enzyme work was carried out.

This research was carried out under the tenure of a fellowship in the Department of Medical Research in the University of Toronto.

REFERENCES.

Chibnall and Channon (1927, 1). *Biochem. J.* **21**, 225.
—— —— (1927, 2). *Biochem. J.* **21**, 233.
—— —— (1927, 3). *Biochem. J.* **21**, 1112.
Grimbert and Bailly (1915). *Compt. Rend. Acad. Sci.* **160**, 207.
Karrer and Salomon (1926). *Helv. Chim. Acta*, **9**, 3.
Kay and Lee (1931). *J. Biol. Chem.* **91**, 135.
King (1932, 1). *Biochem. J.* **26**, 586.
—— (1932, 2). *Biochem. J.* **26**, 292.
Levene (1921). *Physiol. Rev.* **1**, 327.
—— and Rolf (1919). *J. Biol. Chem.* **40**, 1.
Rae, Kay and King (1934). *Biochem. J.* **28**, 143.
Tutin and Hahn (1906). *J. Chem. Soc.* **89**, 1749.
Willstätter and Lüdecke (1904). *Ber. deutsch. chem. Ges.* **37**, 3753.

XXIII. ON METHODS OF DETERMINING THE DEGREE OF CALCIUM DEFICIENCY OF SHEEP.

By ALLAN HENRY HECTOR FRASER, WILLIAM GODDEN and DOUGLAS WILLIAM AUCHINACHIE.

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(*Received November 11th, 1933.*)

The effect of lime and cod-liver oil supplements on the growth and serum-Ca of sheep fed on a Ca-deficient diet has been previously discussed by two of us [Auchinachie and Fraser, 1932].

The present communication records further experiments on the same subject in which Ca-balance determinations over a period were combined with blood analyses and clinical observations on the sheep. It emphasises the possible fallacies which may arise from any single method of investigation.

Four groups of five young sheep of similar age and size were fed the following basal ration:

Cooked maize	1 part	2 lbs. per diem.
Crushed oats	1 ,,	
Bran	1 ,,	
Dried distiller's grains	1 ,,	
Turnips	to 10 lbs. per head daily	
Oat straw	to ½ lb.	,,
Water and salt (NaCl) *ad lib.*		

This was supplemented in the various groups as under:

Group I. Nothing.
II. 2·5 ml. cod-liver oil per head daily.
III. 2·2 lbs. of chalk per 100 lbs. concentrates.
IV. 2·2 lbs. of chalk per 100 lbs. concentrates + 2·5 ml. cod-liver oil per head daily.

The relative intakes of CaO on these diets are shown in Table I. Experimental feeding began on 27th October and continued until 24th May, a period of 209 days. The feeding pens were inside, covered, concrete-floored and bedded with moss litter.

Results.

Clinical.

The growth curves of the four groups are shown in Fig. 1. For a period of 84 days there was no differentiation in growth-rate. Subsequently growth-rate diminished in the basal group. In confirmation of previous work, a combined supplement of chalk and cod-liver oil had a greater effect on growth than either chalk or cod-liver oil alone; but in contrast to earlier findings the beneficial effect of chalk alone was greater than that of cod-liver oil alone [Auchinachie and Fraser, 1932].

The group food consumption was unequal. The question therefore arises as to how much of the basal group's growth retardation was due to decreased appetite, and how much to inefficient utilisation of the food consumed. The question can be answered by consideration of Fig. 2, where the weight of dry matter consumed per month in each group is plotted against time. The food consumed by group IV

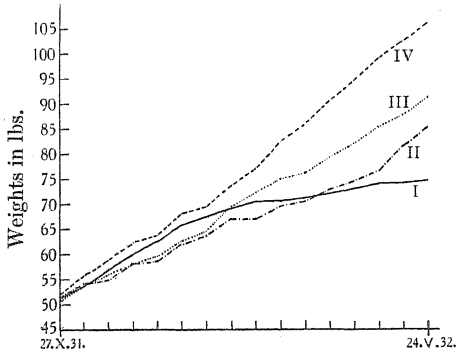

Fig. 1.

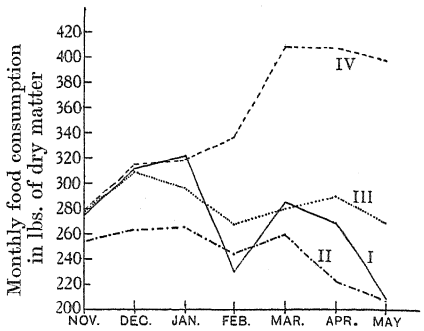

Fig. 2.

Fig. 1. Live weight increase (1931–32).

Group I. Basal. ————. Group II. Basal + C.L.O. —·—·—.
Group III. Basal + $CaCO_3$ ·····. Group IV. Basal + $CaCO_3$ + C.L.O. — — —.

Fig. 2. Food consumption.

Group I. ————. Group II. —·—·—. Group III. ·····. Group IV. — — —.

is seen to be almost double that of any other group. It may therefore be assumed that the omission of either chalk or cod-liver oil markedly decreased appetite. The relative amounts of food consumed during the balance experiments were slightly different owing to idiosyncracies of the individual animals and somewhat differing responses to cage environment.

One effect of the deficiencies of the basal ration was therefore to decrease appetite and food consumption. The utilisation of the food consumed was also inefficient, since while group I consumed more food than group II, its final average weight was nevertheless substantially less.

Balance determinations.

Balance determinations were made on one sheep from each group towards the close of the experiment when group differentiation was marked. The sheep were confined in metabolism cages over a period of 13–16 days. N, CaO and P_2O_5 balances were determined, and the results are shown in Table I. On the basal ration the daily intake of N was 12·18 g., of CaO, 2·10 g. and of P_2O_5, 7·20 g. Of this intake the sheep retained some 4 g. of N, some 0·08 g. CaO and some 1·4 g. P_2O_5. The sheep was therefore practically in equilibrium as far as calcium was concerned.

The chalk supplement increased the CaO intake to 7·20 g. It raised the CaO balance to 1·0 g., at the same time halving P_2O_5 retention and leaving the N balance unaffected.

Cod-liver oil raised the positive CaO balance to 1 g., but at the same time raised the P_2O_5, this latter effect being precisely opposite to that of chalk.

Table I. *Results of balance experiments with individual sheep.*

Group	Ration and average daily intakes (g.)	Average daily excretions: Nitrogen Urine g.	Nitrogen Faeces g.	CaO Urine g.	CaO Faeces g.	P_2O_5 Urine g.	P_2O_5 Faeces g.	Average daily balances: N g.	CaO g.	P_2O_5 g.
I	Basal N, 12·18; CaO, 2·10; P_2O_5, 7·20	4·32	3·79	0·0045	2·02	0·60	5·22	4·07	0·08	1·38
II	Basal + C.L.O. N, 14·57; CaO, 2·48; P_2O_5, 8·43	4·97	4·62	0·0044	1·46	3·38	2·84	4·98	1·02	2·21
III	Basal + Chalk N, 12·18; CaO, 7·20; P_2O_5, 7·20	4·46	3·58	0·0049	6·18	0·04	6·54	4·14	1·02	0·62
IV	Basal + Chalk + C.L.O. N, 15·56; CaO, 8·16; P_2O_5, 7·89	6·91	4·12	0·0211	6·82	1·04	6·07	4·53	1·32	0·78

Chalk and cod-liver oil together raised the CaO balance to the highest level, namely 1·3 g., and decreased P_2O_5 retention.

The relative excess of P_2O_5 retained in groups I and II raises an interesting problem. In group I with calcium in equilibrium there was a daily retention of 1·38 g. of P_2O_5. Obviously this could not have been deposited in the bones in the normal manner as calcium phosphate, and yet osseous tissue is the only large storage depot for phosphorus in the body. There is no evidence in the experimental data to elucidate this point, but further investigations are in progress along these lines.

Blood analysis. The results of periodical serum-Ca and blood-inorganic P determinations are shown in Table II. The changes in plasma-phosphatase have been previously recorded [Auchinachie and Emslie, 1933].

Table II.

Group	October Ca	October P	December Ca	December P	February Ca	February P	March Ca	March P	April Ca	April P
1. Basal	11·55	4·77	9·41	5·88	7·01	8·40	6·29	7·50	6·36	7·59
2. Basal + C.L.O.	11·75	5·19	10·61	5·46	10·52	6·54	10·00	8·17	10·44	7·03
3. Basal + $CaCO_3$	11·68	5·28	10·73	5·12	9·00	5·01	7·75	8·93	7·31	7·12
4. Basal + $CaCO_3$ + C.L.O.	11·04	5·27	10·98	5·77	10·91	6·10	10·96	7·99	11·60	7·43

The figures show that serum-Ca fell to extremely low figures in groups I and III, but that C.L.O. retained it at a comparatively normal level.

Discussion.

The effect on growth of lime and cod-liver oil supplements to a Ca-deficient ration here recorded confirm previous work. The results of the blood analysis and balance determinations require further discussion.

Consideration of Table II shows that the average serum-Ca figure for group I had fallen by April to 6·36 mg., an abnormally low figure. That for group III was 7·31 mg., also abnormally low. On the contrary the figure for group II, 10·44 mg., was within the normal range and only slightly lower than the figure for group IV, 11·60 mg.

The logical interpretation of these figures is that the calcium deficiency of the basal ration lowered the serum-Ca of the basal group; that a chalk supplement had little effect in raising it, but that cod-liver oil, either alone or in combination with chalk, restored it to a normal level. It might therefore be assumed that cod-liver oil was more effective than chalk in restoring a normal calcium balance, and

that cod-liver oil alone was almost as effective in this respect as when combined with chalk.

This interpretation of the results of serum-Ca determinations does not correspond with the growth differentiation of the groups or with the findings of the balance experiments. Consideration of Table I shows that a chalk supplement was as effective as cod-liver oil in increasing the positive CaO balance, and that chalk and cod-liver oil in combination were more effective than either alone; results which are in general conformity with the growth differentiation of the four groups.

The indications of the state of calcium metabolism obtained respectively from blood analysis and balance determinations do not agree. The same is true of phosphorus metabolism. Thus in April the figures for blood-inorganic phosphorus were closely similar in all four groups (Table II), whereas there were wide differences in P_2O_5 retention (Table I) at the same period.

While the serum-Ca figures are averages for all five sheep in each group, the balance determinations were done on single sheep and are not therefore strictly comparable. A more reliable guide is to consider the serum-Ca figures for the individual sheep from blood samples drawn at approximately the same dates as those on which their calcium balances were determined (Table III).

Table III.

Group	Index no. of sheep	Av. daily balance g. CaO	Serum-Ca mg./CaO ml.
I. Basal	78	0·08	5·85
II. „ +C.L.O.	27	1·02	10·70
III. „ +Chalk	49	1·02	8·35
IV. „ + „ +C.L.O.	35	1·32	12·00

From Table III it is again apparent that cod-liver oil had a relatively greater effect in raising the level of serum-Ca than in increasing calcium retention. It is also evident that a chalk supplement has a more favourable influence on calcium retention than the serum-Ca figures would suggest.

On the other hand the association of calcium equilibrium with a serum-Ca figure of half the normal value is unexpected. It probably results from minimum Ca excretion owing to the low serum-Ca level. It follows that balance determinations alone might, under certain conditions, give a misleading indication of normality.

From the results recorded in this communication it is evident that growth rate and clinical observation remain the soundest method of determining the effects of supplements added to a calcium-deficient ration. Blood analysis, particularly, cannot be accepted as a satisfactory substitute, until the factors governing serum-Ca level are more perfectly understood. A calcium dietary deficiency undoubtedly lowers the serum-Ca level, at least in sheep, but the effect of different factors in again raising it is not necessarily correlated with their effect on growth or on calcium retention.

Summary.

1. Supplements of chalk or cod-liver oil, fed to sheep on a calcium-deficient ration, resulted in improved growth rate and health, a combined supplement of chalk and cod-liver oil being more effective than either alone.

2. The arrest of growth in the basal, Ca-deficient group was due partly to decreased food consumption and partly to inefficient utilisation of the food consumed.

3. The Ca-deficient ration lowered the serum-Ca, while supplements of chalk or cod-liver oil tended to retain it at a normal level. In this respect cod-liver oil was more effective than chalk.

4. Balance determinations showed that the basal group was in calcium equilibrium; that both chalk and cod-liver oil produced a positive Ca balance; and that their effects were additive.

5. The effect of cod-liver oil in retaining a normal serum-Ca level was much greater than its effect in increasing Ca retention or rate of growth.

REFERENCES.

Auchinachie and Fraser (1932). *J. Agric. Sci.* **22**, 560.
—— and Emslie (1933). *Biochem. J.* **27**, 351.

XXIV. THE GLYOXALASE ACTIVITY OF TISSUES.

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The authors have already investigated [Jowett and Quastel, 1933] the glyoxalase activity of red blood corpuscles. A study has now been made of the glyoxalase activity of tissues with the use of tissue-slices.

An interesting finding, which former workers have not made clear, is that, owing to the high glyoxalase activity of tissues, diffusion of methylglyoxal sets a limit to the activity shown by tissue-slices of the usual thicknesses. The glyoxalase activity is proportional not to the weight of the tissue-slices, but to their surface areas. The observed activity depends on the diffusion coefficient of methylglyoxal in tissues as well as on the intrinsic activity of the cells.

This additional variable makes the designing and interpretation of experiments more difficult. Nevertheless we have been able to obtain a little further information on the part played by methylglyoxal in glycolysis.

Methods.

Rates of glyoxalase action have been measured, as in our former work [Jowett and Quastel, 1933], by the manometric method of Warburg. Methylglyoxal solution, contained in a side-tube, is added to the Ringer's solution after equilibration of the manometers at 37°. Manometric readings are begun 5–10 minutes later.

Because comparatively large weights of tissue are used (usually of the order 5–12 mg., no micro-balance being available), the rate of gas output is high, and measurements cannot be continued for long before changes in the methylglyoxal concentration become appreciable. The high rates however permit fairly accurate measurements of the velocity to be made over a short period.

Methylglyoxal was made by the method of Riley *et al.* [1932], a practically neutral solution being obtained[1]. We have assumed that the gas-output in our anaerobic experiments corresponds to formation of lactic acid. The gas output is completely thermolabile.

The anaerobic glyoxalase activity of tissue-slices.

In Table I are given the results of experiments on the glyoxalase activity of tissue-slices under anaerobic conditions. The only substrate added is methylglyoxal, and the proportion of the gas output due to glycolysis is very small.

Slices of several thicknesses have been employed in each experiment. It is found that the value of $Q^{N_2}_{M}$ is greater the thinner the slice, and even with the thinnest slices used there is no evidence that $Q^{N_2}_{M}$ has reached a limiting value.

[1] For the methylglyoxal used in our former work we are indebted to Prof. C. Neuberg.

Table I. *Anaerobic glyoxalase activity of tissue-slices of varying thicknesses.*

At 37°. $N_2+5\ \%\ CO_2$.
Ringer's solution containing $0{\cdot}025\,M$ $NaHCO_3$.
Initial methylglyoxal concentration $M/100$. $v_F=3{\cdot}2$ ml.
Measurements relate to the period about 7–25 minutes after addition of methylglyoxal.
$Q_M^{N_2}$ = mm.^{3} CO_2 evolved per hr. per mg. dry weight.
$S_M^{N_2}$ = mm.^{3} CO_2 evolved per hr. per cm.^{2} of surface.

Tissue	Thickness of slice mm.*	$Q_M^{N_2}$	$S_M^{N_2}$
Guinea-pig kidney	0·22	105	240
	0·27	86	235
	0·41	74	(295)
	0·47	51	245
Guinea-pig liver	0·43	29·5	127
	0·43	31·0	133
	0·53	22·8	122
Jensen rat sarcoma	0·27	38·5	(103)
	0·37	34·5	128
	0·37	33·5	123
	0·42	30·5	128
Jensen rat sarcoma	0·32	33	116
	0·37	30	111
	0·45	25·5	116
	0·66	17·5	117
Rat kidney	0·17	110	191
	0·20	96	196
	0·27	70	191
	0·38	61	(235)

* The thicknesses of the slices are calculated by the approximate assumption that the volume of a slice in mm.^{3} is five times its dry weight in mg.

On the other hand S, the rate per unit surface[1], is constant within the experimental error. (The data have not been selected, and some irregularities are evident.)

These findings show that methylglyoxal in appreciable quantity is penetrating only a short distance into the slices. Only a thin outer layer of the slices contributes appreciably to the metabolism; hence the proportionality to the surface area. If sufficiently thin slices were studied, $Q_M^{N_2}$ should rise to a value almost independent of the thickness, and S should decrease as slices below the limiting thickness are reached. Our experiments have not been made with slices sufficiently thin to show these limiting phenomena, and consequently we have no direct method of deducing from our data either the maximum value of $Q_M^{N_2}$ or the limiting thickness.

It is clear that our values of $Q_M^{N_2}$, in common with those of former workers, do not measure the glyoxalase activity of tissue cells supplied with a definite concentration of methylglyoxal but give a mean activity averaged over all the cells of the tissue-slice, some of these cells obtaining practically no methylglyoxal.

The values of S, the activity per unit surface, are however characteristic for each tissue. S will be a function both of the rate at which cells can transform methylglyoxal into lactic acid and of the diffusion coefficient of methylglyoxal. Under certain conditions [Jowett, 1934] the value of S is given by the equation

$$S=\sqrt{2ADCo} \qquad \text{.....(1),}$$

[1] S is defined in Table I. In calculating surface both sides of the slice are counted, and the area due to the edges has been neglected.

where A is the amount of methylglyoxal consumed per unit volume of tissue adequately supplied with it[1], D is the diffusion coefficient of methylglyoxal in the tissue, and Co is the concentration of methylglyoxal in the outer medium.

The diffusion coefficient, D, may not change much from one tissue to another, and variations in S may be chiefly caused by differences in A, which measures the capacity of tissues to transform methylglyoxal when supplied with it. From the data in Table II it seems likely that A, and therefore the maximum $Q_M^{N_2}$, is higher for kidney than for liver and tumour tissue. The figures in Table II each relate to tissue from a different animal and show that S for any one tissue is fairly constant from one individual animal to another.

Table II. *Anaerobic glyoxalase activity of tissues.*

Conditions as in Table I.

Tissue	$S_M^{N_2}$
Guinea-pig kidney	240, 240, 232, 271
Guinea-pig liver	127, 130
Jensen rat sarcoma	126, 115
Rat kidney	193, 175

In Table III are given the highest values of $Q_M^{N_2}$ which we have observed for various tissues. In each case, the figures provide a lower limit to the maximum value of $Q_M^{N_2}$. The values found by other workers are given for comparison. Conclusions as to the relative activity of glyoxalase in the tissues

Table III. *Anaerobic glyoxalase activity of tissue-slices.*

Conditions as in Table I.

Tissue	Highest observed value of $Q_M^{N_2}$
Rat spleen	50
,, testis	60
,, brain	46
,, sarcoma (Jensen)	38
,, kidney	110
Guinea-pig brain	107
,, testis	54
,, kidney	105
,, liver	40
,, lung	40

Measurements by other authors.

Tissue	$Q_M^{N_2}$	Author
Rat liver	24	Warburg *et al.* [1924]
Flexner rat carcinoma	29	,,
Brain (rat?)	25–50	Meyerhof [1925]
Liver (rat?)	30	,,
Diaphragm (rat?)	15	,,
Rat liver	55	Rosenthal [1930]

cannot be drawn from these data. The thickness of the slices varies considerably, and furthermore the limiting thickness for maximum activity must also vary. We cannot yet estimate how far we are from the maximum values. For human red blood corpuscles we found [Jowett and Quastel, 1933] values of $Q_M^{N_2}$ about 55–75, which are presumably maximum. Other cells may be expected to show higher limiting values, and values up to 200 are quite conceivable.

[1] Regions partially but not adequately supplied are neglected.

The observed anaerobic glyoxalase activity of most tissues, using tissue-slices in 0·01 *M* methylglyoxal, falls off fairly rapidly as time passes. This is illustrated in Fig. 1, where the rates of gas output are plotted against time, taking the initial rate in each case arbitrarily to be 100. The first measurement of rate relates to a time about 10 minutes after methylglyoxal is added. While the velocity-time curves are similar for duplicate slices of any one tissue from the same individual animal, there is much variation from one animal of the same species to another. The variability from one tissue to another shows that the effect can only be due in a minor degree to the diminution in substrate concentration with time.

The behaviour of red blood corpuscles is shown in Fig. 2. The rate in 0·01 *M* methylglyoxal falls off slowly. In 0·02 *M* methylglyoxal the decrease in rate is greater. The decrease in 0·005 *M* methylglyoxal is chiefly due to the low substrate concentrations which are soon reached as the methylglyoxal is consumed. The experiment shows that, in a case where diffusion should be adequate to saturate the enzyme system with methylglyoxal, the maximum value of $Q_M^{N_2}$ is reached at a low methylglyoxal concentration. Raising the methylglyoxal concentration evidently has a toxic effect on the enzyme system, for the initial value of $Q_M^{N_2}$ is lower in the higher concentrations, and the rate of decrease in $Q_M^{N_2}$ is greater.

That tissue-slices are damaged by methylglyoxal is suggested by their change in appearance, on which Kisch [1932] has already remarked. Tissue-slices usually become tougher and firmer, and frequently swollen and opaque, after exposure to 0·01 *M* methylglyoxal in Ringer's solution. Slices of brain become fragile to handle. Loss of pigment, which normally takes place with slices of spleen and liver, is prevented. The changes in appearance are more rapid in higher methylglyoxal concentrations; in concentrations below 0·01 *M* they are less marked.

From these changes, it would not be surprising if the diffusion coefficient of methylglyoxal in tissues altered as time passed. The behaviour of rat testis, shown in Fig. 3, may be due to such changes. The observed glyoxalase activity goes through a maximum at the higher concentrations, which may be due to an initial lowering in the ability of methylglyoxal to diffuse, which then disappears as the membranes are further damaged. The diffusion of sodium lactate through muscle shows analogous changes [Eggleton *et al.*, 1928].

Fig. 4 illustrates the behaviour of guinea-pig liver. In this experiment 0·01 *M* methylglyoxal apparently had little toxic effect on the enzyme system, while higher concentrations exerted progressive inhibitory action. Both Figs. 3 and 4 illustrate the tendency for high methylglyoxal concentrations to produce higher apparent glyoxalase activity. We consider that this effect is due to a thicker layer of tissue being supplied with substrate.

It is only in the case of red blood corpuscles that we can safely attribute the effects of raising the methylglyoxal concentration entirely to toxic action. With tissue-slices we have as additional factors: (1) the better supply of methylglyoxal to the tissue, and (2) possible changes in the diffusion coefficient of methylglyoxal.

In carrying out experiments on the effect of varying conditions on the observed glyoxalase activity of tissue-slices, two methods have been used:

(1) The measurement of activity per unit area of surface (S).

(2) The use of tissue-slices of equal thickness, activity then being stated per unit weight (in terms of $Q_M^{N_2}$). By choosing slices carefully, and then cutting each slice into several smaller ones, which are distributed among the several manometric vessels, it is possible to obtain fairly reproducible behaviour, with

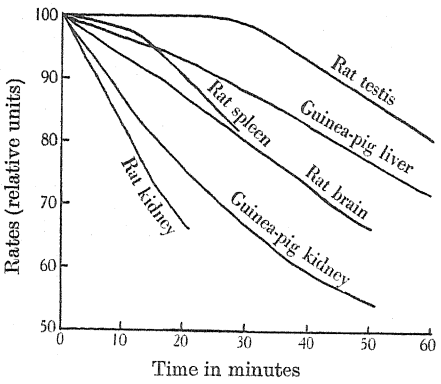

Fig. 1. Anaerobic glyoxalase activity of tissue-slices as a function of time.

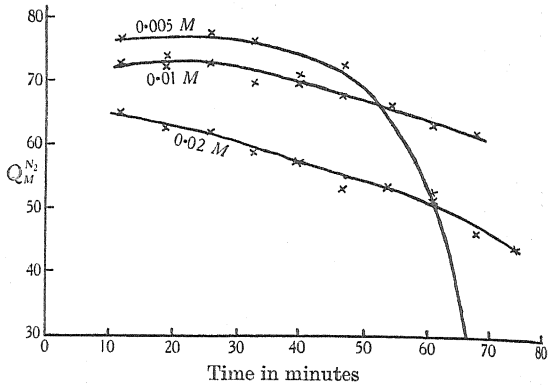

Fig. 2. Glyoxalase activity of human erythrocytes, at several methylglyoxal concentrations.

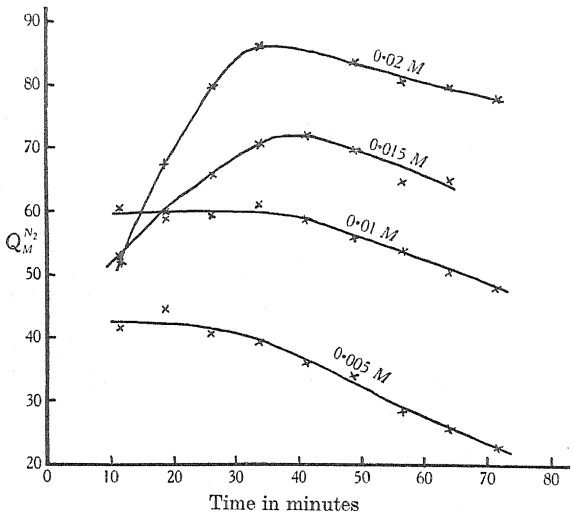

Fig. 3. Glyoxalase activity of rat testis at various methylglyoxal concentrations.

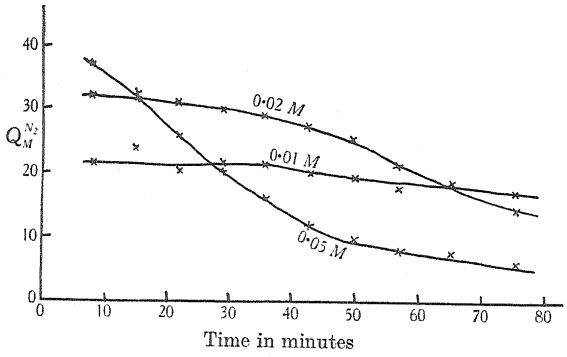

Fig. 4. Glyoxalase activity of guinea-pig liver at various methylglyoxal concentrations.

occasional irregularities. This method, which was used in obtaining the data of Figs. 1, 3 and 4, is applicable in many cases where rectangular slices of reasonable size—for measurement of area—are hard to obtain.

From what has already been said, it should be clear that changes in the observed activity do not necessarily correspond with equal changes in the intrinsic glyoxalase activity of tissues. An additional point will be noted here. If the intrinsic activity falls, the methylglyoxal will penetrate further into the slice (no change in diffusion coefficient being supposed to take place), and as a result the observed change in activity will not be so great as the change in intrinsic activity. When equation (1) holds good, S will change proportionally to the square root of A, if D and Co are constant. The observed values of $Q_M^{N_2}$ will similarly change proportionally to the square root of the maximum $Q_M^{N_2}$, provided that the slices are incompletely saturated with substrate.

According to equation (1) S should vary as the square root of the concentration of methylglyoxal. At very low concentrations of methylglyoxal, according to Jowett [1934], S should vary instead proportionally to the concentration. That is, S varies as c^n, where n changes from 1 at low values of c to 0·5 at high values. Toxic effects are not considered. Measurements have been made of the values of S for guinea-pig kidney at a number of methylglyoxal concentrations; the data, shown in Fig. 5, refer to two different experiments. They appear to fit the linear equation best in dilute solutions and the square-root relation better in stronger solutions. The values of S are however not very suitable for testing the equations. In dilute solutions the substrate concentration is falling off rapidly with time, and in the stronger solutions toxic effects enter.

The influence of glucose on glyoxalase action.

If methylglyoxal is an intermediary in glycolysis, when methylglyoxal and glucose are both presented to a tissue competition between the two substances should occur. More strictly, the competition would be between added methylglyoxal and that produced from glucose. Addition of glucose should not increase the rate of production of lactic acid by a tissue saturated with added methylglyoxal. On the other hand, if glycolysis and glyoxalase action are quite independent, the rates of these processes may proceed independently and additively.

Table IV. *Anaerobic production of lactic acid from glucose and methylglyoxal by tissue-slices.*

Conditions as in Table I. The values relate to a period about 6–20 minutes after methylglyoxal is added. Where glucose is added, its concentration is 0·2 %, and it is present in the Ringer's solution before raising the temperature to 37°.

Tissue	No. of experiments	Mean values of $Q_M^{N_2}$: Substrate absent	Glucose	Methylglyoxal	Methylglyoxal + glucose
Rat spleen	2	2·0	9·5	43	46
Rat testis	4	0·5	8	41·5	40
Rat brain*	4	1·5	16	42·5	58
Guinea-pig testis	2	4·5	11	44	48
Guinea-pig kidney	2	—	8	99·5	98
Jensen rat sarcoma*	2	2·5	27	34	43·5

* The measurements of $Q_M^{N_2}$ in the absence of substrate and in presence of glucose were made in these cases before methylglyoxal was added to obtain the other values for $Q_M^{N_2}$. The effect is to exaggerate relatively the former figures.

Experimentally the difficulty is to saturate tissue-slices with methylglyoxal, and in the experiments given in Table IV we do not know how far this saturation is attained. Competition, if it is taking place at all, may only take place in a small portion of the tissue-slice, the central portions being supplied only with glucose.

The data suggest that some competition is taking place, except in the case of rat brain, and they therefore appear to support to some extent the view that methylglyoxal is an intermediary in glycolysis. Other interpretations are not excluded. We consider that further experiments on this subject should be restricted to tissues, such as leucocytes, which can be adequately supplied with methylglyoxal.

The effect of oxygen on glyoxalase activity.

The glyoxalase activity of human erythrocytes is less in oxygen than anaerobically [Jowett and Quastel, 1933]. With regard to tissue-slices, Meyerhof [1925] has stated that oxygen has no effect on the glyoxalase activity of brain and attributes the lower apparent activity of diaphragm and liver in oxygen to depression of glycolysis.

Respiration and glycolysis are complicating factors in experiments with tissue-slices. By omitting glucose from our Ringer's solution we consider we have avoided appreciable glycolysis in our experiments (except possibly with liver).

As regards respiration, we have adopted, in a first series of experiments, the assumption that respiration proceeds unchanged in presence of methylglyoxal and have corrected the observed manometric changes in oxygen on this basis, employing either measurements of our own for the manometric correction due to respiration, or data such as those of Dickens and Greville [1933].

We have found that the oxygen uptake of slices of guinea-pig liver and kidney is not affected immediately by 0·01 *M* methylglyoxal; appreciable inhibition develops in 30–60 minutes. We have therefore some evidence that Q_{O_2} is unaffected by methylglyoxal. That the R.Q. is unaffected has been assumed without direct experimental evidence.

The observed percentage effects of oxygen on the rate of glyoxalase action of tissue-slices are shown in Table V, the effects before and after correction for

Table V. *Effect of oxygen on observed glyoxalase activity of tissue-slices.*

Conditions as in Table I, except that activity is calculated per unit weight of tissue. Measurements relate to a period 6–20 minutes after addition of methylglyoxal. Rates in $O_2 + 5\ \%\ CO_2$ compared with those in $N_2 + 5\ \%\ CO_2$.

Tissue	No. of experiments	Mean percentage effect of oxygen	
		Uncorrected	Corrected
Guinea-pig liver	5	−18	− 9
,, kidney	4	−16	− 8
Rat testis	4	+ 4	+ 9
,, brain	3	+ 9	+11
,, spleen	3	−24	−14
,, kidney	1	−25	−14
Jensen rat sarcoma	2	−11	0

respiration being given. Evidently the initial effect of oxygen on glyoxalase activity is not large. As time passes, the apparent inhibitory effect of oxygen increases. This is shown in Figs. 6 and 7, in which are given the rates in oxygen (uncorrected for respiration) relative to those in nitrogen as a function of time.

The initial ratio is arbitrarily taken as 100 in each case. In every case the ratio falls off as time passes[1]. If an inhibitory effect of oxygen is not present at first (Table V), it appears to develop as time passes. It is interesting to note that

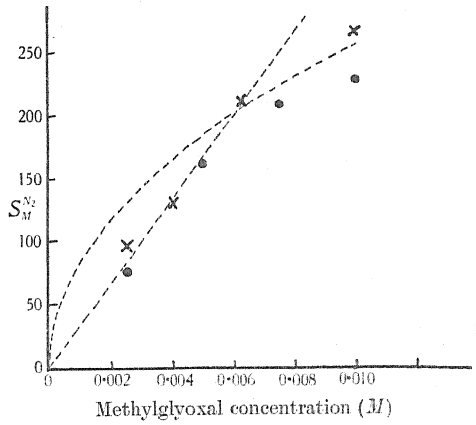

Fig. 5. Glyoxalase activity of guinea-pig kidney per unit surface.

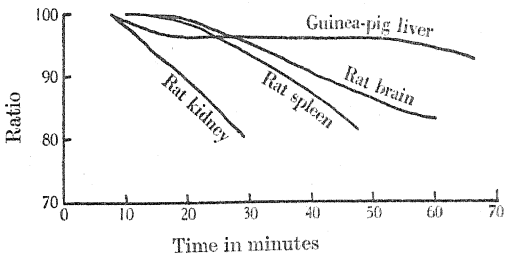

Fig. 6. Ratio of aerobic to anaerobic glyoxalase activity for various tissues.

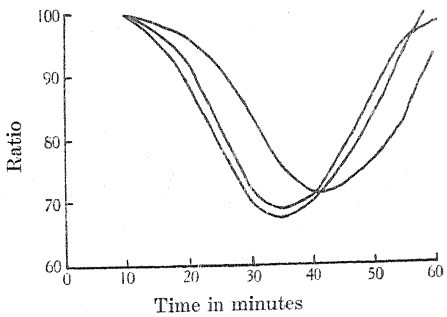

Fig. 7. Rat testis. Ratio of aerobic glyoxalase activity to anaerobic glyoxalase activity.

rat testis shows anomalous behaviour (Fig. 7), the ratio passing through a minimum and then rising again.

In a second series of experiments we have measured aerobic glyoxalase action by the method of Negelein [1925], in which the decrease of bicarbonate concen-

[1] Data for Jensen rat sarcoma are not available.

tration during the experimental period is a measure of acid production. In this series the activity per unit surface of the tissue-slices has been measured. The data obtained are given in Table VI. In these experiments the p_H and bicarbonate concentration change considerably, and hence in each experiment the sizes of slices have been chosen so that the total glyoxalase action in the nitrogen-containing vessel is nearly equal to that in the oxygen-containing vessel.

Table VI. *Anaerobic and aerobic glyoxalase action of tissue-slices.*

$M/100$ methylglyoxal. N_2 or O_2 containing 1·5 % CO_2.
3·2 ml. Ringer's solution containing 0·0073 M $NaHCO_3$.

Tissue	Experimental period mins.	$S_M^{N_2}$	$S_M^{O_2}$	Percentage effect of oxygen
Guinea-pig kidney	49	168	132	−21
,, liver	50	84	72	−14
,, ,,	34	80	66	−17
,, ,,	60	76	50	−34
,, ,,	50	94	69	−26
,, ,,	46	91	70	−23
,, ,,	66	80	62	−23
Cat brain*	37	—	—	−20
,, *	30	—	—	−17

* In the case of cat brain the activity per unit weight was measured.

The experiments show a quite definite inhibitory effect of oxygen on the acid production. Since lactate is being produced by glyoxalase action, in experiments of any length some lactate will be removed by oxidation. We consider, however, that the effect of lactate oxidation on our results will be small.

The real action of oxygen on glyoxalase activity of cells will, according to equation (1), be nearly twice the effect observed on the activity of tissue-slices[1]. Accordingly, the effect of oxygen on the activity of cells may be greater than on that of erythrocytes, for which it amounts to about 20 %.

In considering the effect of oxygen on glyoxalase activity in relation to its possible bearing on glycolysis, it must be remembered that the effect of oxygen may be a function of the methylglyoxal concentration. As methylglyoxal combines with glutathione, the coenzyme of glyoxalase [*cf.* Jowett and Quastel, 1933], a high methylglyoxal concentration may partially protect glutathione from oxidation, and the effect of oxygen on glyoxalase activity may therefore be less at high methylglyoxal concentrations than at low ones. This expectation appears to be realised in the case of human erythrocytes (Table VII).

Table VII. *Glyoxalase activity of human erythrocytes.*

5 % CO_2. $NaHCO_3$ 0·025 M. 0·2 % glucose present. Cells well washed with saline.

Exp. No.	Methylglyoxal 0·02 M			Methylglyoxal 0·005 M		
	$Q_M^{N_2}$	$Q_M^{O_2}$	$Q_M^{O_2}/Q_M^{N_2}$	$Q_M^{N_2}$	$Q_M^{O_2}$	$Q_M^{O_2}/Q_M^{N_2}$
1	62·5	55·5	0·88	67·5	56	0·83
2	63·5	55	0·86	75	58·5	0·78
3	46	44·5	0·97	59·5	46·5	0·78
		Mean	0·90			0·80

If the greater inhibitory effect of oxygen on glyoxalase activity is a general phenomenon at low methylglyoxal concentrations, the supposition that methyl-

[1] Because A, or the maximum $Q_M^{N_2}$, varies as S^2.

glyoxal is an intermediary in glycolysis goes some way to explain the Pasteur phenomenon. Methylglyoxal as an intermediary will be present in tissues only in low concentrations, or glycolysis would proceed much faster than it does.

It may be remarked that the Pasteur phenomenon, *i.e.* the diminution of glycolysis in oxygen, is definitely in need of explanation. The view advanced by Meyerhof and Warburg, that in oxygen tissues can resynthesise lactic acid to carbohydrate, has so far been substantiated only for one mammalian tissue, liver [Cori and Cori, 1929]. Yet liver is the one tissue in which it is doubtful, from the data of Dickens and Greville [1933], whether glycolysis is affected by oxygen.

Lipmann [1933] has put forward the interesting suggestion that the glycolytic ferment may be reversibly oxidised, the oxidised form being inactive[1]. The suggestion rests principally on experiments with dyestuffs which are considered to act solely as oxygen carriers. It is to be remembered, however, in considering Lipmann's experiments that a number of dyestuffs are toxic to ferments [*cf.* Yabusoe, 1925; Quastel, 1932], and it is possible that this toxicity is lost on reduction of the dyestuffs to their leuco-forms.

Glyoxalase activity in relation to glycolysis.

Experiments on muscle extracts have led Embden *et al.* [1933] to propose, and Meyerhof and Kiessling [1933] to support, a mechanism for glycolysis in which methylglyoxal has no place. Their work cannot however permit us to ignore the evidence that methylglyoxal is, in some tissues at least, a probable precursor of lactic acid. Many facts support or are in accordance with such a view.

(1) Tissues treated so as to inhibit glyoxalase activity produce methylglyoxal from hexosediphosphate [Neuberg and Simon, 1933] and from glyceraldehyde-phosphoric acid [Barrenscheen and Beneschovsky, 1933].

(2) All tissues investigated, with the exception of pancreas, can transform methylglyoxal into lactic acid.

(3) The kinetics of glyoxalase action satisfy the conditions required for participation in glycolysis.

(*a*) The rate of transformation of methylglyoxal into lactic acid by tissues is high and should proceed as rapidly as glycolysis at quite low methylglyoxal concentrations. The best evidence for this is provided by the present work. It had previously been in doubt whether the glyoxalase activity of tumour tissue was sufficiently high.

(*b*) The activity of glyoxalase extends over a range of p_H similar to or greater than that of the glycolytic enzyme-system [Kuhn and Heckscher, 1926; Lohmann, 1932].

(*c*) Glyoxalase action is only inhibited by substances which also affect glycolysis to an equal or greater extent. The actions of fluoride [Lohmann, 1933], iodoacetic acid [Dudley, 1931; Lohmann, 1933], and narcotics [Meyerhof, 1925], may be mentioned in this connection. Pancreatic extracts affect both enzyme systems [Harrison and Mellanby, 1930; Giršavičius, 1933] but quantitative comparisons have not been made.

(*d*) It also appears, from the present work, that the effect of oxygen on glycolysis is paralleled to some extent by its effect on glyoxalase action.

It must be admitted that the methylglyoxal theory meets with certain difficulties. Lohmann has shown [1932] that muscle extracts possessing no

[1] This suggestion is formally similar to the view already expressed by the authors [1933] that a partial explanation of the Pasteur phenomenon may be found in the reversible oxidation of glutathione—the co-enzyme of glyoxalase.

glyoxalase activity can break down glycogen to lactic acid. This proves that at least one tissue—or rather one tissue extract—has a mechanism of glycolysis not involving methylglyoxal. Many facts however favour the view that different tissues may have glycolytic mechanisms differing in detail, and even that one tissue may have several mechanisms for glycolysis (*cf.* Ashford, 1933). It can be suggested that several paths from carbohydrate to lactic acid may exist in tissues, and that methylglyoxal is not an intermediary in all of them.

The lactic acid produced by glyoxalase action is also apparently not stereochemically identical with that produced by glycolysis. It is not however certain that glycolysis produces only the *d*-form. It is, alternatively, conceivable that the glyoxalase action of undamaged cells at low methylglyoxal concentrations may produce only the *d*-form, for the variability in the rotations observed suggests that toxic effects may be playing a part.

SUMMARY.

1. An investigation of the glyoxalase activity of tissue-slices shows that diffusion of methylglyoxal into the slices limits the rate of reaction. Glyoxalase action is proportional to the surface areas of tissue-slices, not to their weights. The glyoxalase activity of tissues is greater than has been hitherto supposed.
2. It is possible that methylglyoxal and glucose compete as substrates.
3. Oxygen partially inhibits glyoxalase activity in most tissues.
4. Glyoxalase activity is discussed in relation to glycolysis.

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REFERENCES.

Ashford (1933). *Biochem. J.* **27**, 903.
Barrenscheen and Beneschovsky (1933). *Biochem. Z.* **265**, 159.
Cori and Cori (1929). *J. Biol. Chem.* **81**, 389.
Dickens and Greville (1933). *Biochem. J.* **27**, 832.
Dudley (1931). *Biochem. J.* **25**, 439.
Eggleton, Eggleton and Hill (1928). *Proc. Roy. Soc. Lond.* B **103**, 620.
Embden, Deuticke and Kraft (1933). *Klin. Woch.* **12**, 213.
Giršavičius (1933). *Biochem. Z.* **260**, 278.
Harrison and Mellanby (1930). *Biochem. J.* **24**, 141.
Jowett (1934). (In the press.)
—— and Quastel (1933). *Biochem. J.* **27**, 486.
Kisch (1932). *Biochem. Z.* **253**, 373.
Kuhn and Heckscher (1926). *Z. physiol. Chem.* **160**, 116.
Lipmann (1933). *Biochem. Z.* **265**, 133.
Lohmann (1932). *Biochem. Z.* **254**, 332.
—— (1933). *Biochem. Z.* **262**, 152.
Meyerhof (1925). *Biochem. Z.* **159**, 432.
—— and Kiessling (1933). *Biochem. Z.* **264**, 40.
Negelein (1925). *Biochem. Z.* **158**, 121.
Neuberg and Simon (1933). *Ergebn. Enzymforsch.* **2**, 118.
Quastel (1932). *Biochem. J.* **26**, 1685.
Riley, Morley and Friend (1932). *J. Chem. Soc.* 1875.
Rosenthal (1930). *Biochem. Z.* **227**, 354.
Warburg, Posener and Negelein (1924). *Biochem. Z.* **152**, 309.
Yabusoe (1925). *Biochem. Z.* **168**, 227.

XXV. THE EFFECT OF PYOCYANINE ON THE RESPIRATION OF SOME NORMAL TISSUES AND TUMOURS[1].

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Warburg's theory states that the tumour cell suffers from a relative insufficiency of respiratory enzyme, so that even under aerobic conditions the balance of the Pasteur-Meyerhof reaction shows an excess of lactic acid. This suggests that the lack of enzyme might be compensated by the addition of an accessory catalyst. This working hypothesis gains support from the evidence that a similar mechanism actually occurs in nature [Friedheim, 1931]. The *Bacillus pyocyaneus* contains, besides cytochrome, a respiratory ferment similar to that found in higher animals in that it is sensitive to CO and KCN. However, even with free access of molecular oxygen this respiratory enzyme proves insufficient in the absence of assimilable extraneous substances; the oxygen consumption is minimum, the respiratory quotient is almost zero and the oxidation incomplete. Corresponding to those conditions described by Warburg as being characteristic of the tumour cell, *B. pyocyaneus* lives almost as an anaerobic organism even in the presence of air. Under certain conditions it produces the blue pigment, pyocyanine, which, owing to the reversibility of its oxidation and reduction, acts as an accessory oxidation catalyst. In its presence oxygen consumption is increased as much as twenty-four fold and the R.Q. approximates to unity.

It has previously been shown [Friedheim, 1931] that the stimulating effect of pyocyanine on respiration is in no way species-specific but is also to be observed in the red blood corpuscles of rabbits. In the following the effect of pyocyanine on the respiration of some normal and tumour tissues will be described.

Experimental.

Procedure. The Barcroft-Warburg manometric method was employed. Tissue sections 0·2 mm. thick were put with 2 ml. of Ringer's solution into the main chamber of a 10 ml. respiration vessel. For the absorption of CO_2 the inner vessel contained 0·2 ml. of 6 % NaOH, the accessory bulb 0·2 ml. $M/50$ solution of recrystallised pyocyanine. The thermostat was kept at 37·5°. The manometer was shaken at the rate of fifty complete oscillations per minute. The oxygen consumption in Ringer's solution was measured for a first period varying from 30 to 60 minutes. The pyocyanine was then tipped from the accessory vessel into the main chambers, and the observations were continued during a second equal period. This method suffers from the unphysiological condition that the partial pressure of CO_2 is held at approximately zero; but against this drawback stands the great advantage of extraordinary simplicity and the identity of the tissue fragment in the experiment and the controls.

[1] This work has been carried out in the department of Prof. Leonor Michaelis at the Rockefeller Institute for Medical Research, New York. I wish to express my indebtedness and gratitude to Prof. S. Flexner and to Prof. L. Michaelis for the hospitality and the facilities they kindly extended to me.

Tissue sections which were introduced into the previously warmed respiration vessels within a period of less than four minutes after the death of the animal showed the oxygen consumption to be strictly proportional to the time for a period of at least 100 minutes. The deviations to be expected with exclusion of bacterial growth are in the direction of a decrease in respiration and cannot exaggerate any observed increase in respiration. The Ringer's solution contained 0·0025 *M* bicarbonate. In the experiments where sugar was added, a concentration of 0·2 % glucose was used. In those in which phosphate-Ringer was employed, the phosphate concentration was 0·01 *M* and the p_H 7·4.

Results. As can be seen from Tables I–III, the respiration of rat kidney in carbonate-Ringer is either uninfluenced or slightly impeded by pyocyanine; whereas the respiration of testicular tissue, and more so that of tumours, is very

Table I. *Respiration of rat kidney.*

Corrected manometric values for mm.^{3} O_2 absorbed obtained in two periods of equal duration, the first without, and the second with, pyocyanine.

Duration of exp.	30–60 mins.
Thickness of section	0·2 mm.
Glucose concentration	0·2 %
Atmosphere	O_2
Temperature	37·5°
Final pyocyanine concentration	*M*/500
Absorption of CO_2 by 6 % NaOH	

	In carbonate-Ringer 2·5/1000 *M*			In phosphate-Ringer (1/100 *M*) p_H 7·4		
Exp. No.	Without* pyocyanine	With pyocyanine	Difference %	Without pyocyanine	With pyocyanine	Difference %
1	26·0	25·6	− 1·5	18·0	22·1	+22·7
2	19	16·5	−13·15	22	29	+31·8
3	32	26	−12·5	31	31·8	+ 2·5
4	18·5	18·0	− 2·7	25	36	+ 3·6
5	35	31	−11·4	35	37·5	+ 7·1
6	35	37	+ 5·7	50	53·0	+ 6·0
7	30·4	30·7	+ 1·9	54·0	60·2	+11·5
		Mean difference	−0·5 %		Mean difference	+16·8 %

* Mean value of Q_{O_2}, 14·2.

Table II. *Respiration of testicular tissue (rat).*

Corrected manometric values for mm.^{3} O_2 absorbed obtained in two periods of equal duration, the first without, the second with, pyocyanine.

Testicular tubuli carefully dissociated to form only one layer.

Ringer solution	$NaHCO_3$ 2·5/1000 *M*
Duration of exp.	30–60 mins.
Glucose	0·2 %
Final pyocyanine concentration	*M*/500
Atmosphere	O_2
Temperature	37·6°
Absorption of CO_2 by 6 % NaOH	

Experiment No.	Without* pyocyanine	With pyocyanine	Difference %
1	59	67	+30·5
2	43	49	+13·9
3	53	58	+ 9·4
4	59	68	+15·2
5	22	30	+30
		Mean difference	+19·8 %

* Mean value of Q_{O_2}, 9·4.

Table III. *Respiration of tumour tissue and rat testicle in Ringer solution 2·5/1000 M $NaHCO_3$.*

(Conditions the same as in Table I).

		With 0·2 % glucose			Without glucose		
Tissue	Experiment No.	Without pyocyanine	With pyocyanine	Difference %	Without pyocyanine	With pyocyanine	Difference %
Mouse sarcoma S 37	1	23	32·7	+42	35	23·5	−25·7
	2	20	32	+60	39	33	−15·3
	3	39	54	+38·5	25	28	+12
	4	23	62	+170	20	20	0·0
	5	23	31·3	+36	27	24·1	−11
	6	57	78	+36·9			
	7	28	34	+23·5			
	8	29	61·6	+105	26	30·3	+16·5
		Mean values		+63·8			−4 %
Mouse sarcoma S 159	1	16·4	24	+46·3	25	27	+8
	2	19	33	+73·7	23	27	+17·4
	3	18	24	+33·7	18	16	−11
	4	29	39	+37·9			
	5	18	22	+22·2	23	23	0·0
	6	22	32	+45·4	26	28	+7·7
		Mean values		+43·2			+2·4
Human breast carcinoma	1	9	23	+155	7	8	+12·5
	2	22·5	29·6	+31·8	18	18	0·0
	3	10	24	+140			
Spindle-cell sarcoma of humerus	1	23·5	42	+78·8	14	14	0·0
Neuro-blastoma of retina	1	12	27	+125	20	37	+75
Rat testicle (mm.^{3} O_2/hr./mg.)	1	9·02	11·65	+29·7	7·4	7·4	0·0
	2	9·72	12·31	+26·6	7·2	7·2	0·0

considerably enhanced. It is worthy of note that the presence of glucose is essential for the increase of respiration observed with pyocyanine. This is contrary to the findings in the case of bacteria, where the catalytic effect of pyocyanine is exercised on the substance of the cell itself only in the absence of extraneous assimilable substances.

The relative toxicity of pyocyanine and methylene blue.

Harrop and Barron [1928] were the first to show that the respiration of living cells can be increased by the catalytic action of reversible dyestuffs such as methylene blue. These authors found, in accordance with the theory, that the accessory catalyst could only enhance the respiration in cases where there is aerobic glycolysis, *i.e.* where the respiration is insufficient to bring about the disappearance of all lactic acid in the Pasteur-Meyerhof reaction. Our experiments confirm in principle those of Barron [1930], as they show that kidney, a tissue without aerobic glycolysis, is uninfluenced by pyocyanine in its respiration, or if anything, slightly hindered; whereas in tumours with positive aerobic glycolysis the respiration is considerably increased. In accordance with their theoretical considerations the above-mentioned authors expected to find that in the case of testicular tissue, which exhibits aerobic glycolysis, the respiration would be increased on the addition of methylene blue. However, methylene blue

failed to bring about this effect, which, as can be seen from Table II, was brought about by pyocyanine. As the potential range of the two dyestuffs is of the same order of magnitude (E_0 pyocyanine $= -34$ mv. E_0 methylene blue $= +11$ mv. at p_H 7·0), the cause of the dissimilarity in their actions is to be sought in a difference in toxicity. In the case of testicular tissue, the catalytic action seems to be almost exactly compensated by the toxic action. Pyocyanine, probably owing to its small affinity for proteins and high affinity for lipoids, is much less toxic than methylene blue. In the previous experiment with rabbit erythrocytes, pyocyanine caused an increase in respiration of 1250 % (duration of experiment, one hour), a figure which is just above the best values reported by Harrop and Barron [1928] and Michaelis and Salomon [1930] with methylene blue.

In normal rat kidney, which shows practically no glycolysis, the toxicity cannot be masked by any catalytic action. In spite of this fact the average inhibition of respiration brought about by pyocyanine is only 5·8 %, while with methylene blue, according to Harrop and Barron, this amounts to 13·8 %. It is to be emphasised that our experiments were carried out with carbonate-Ringer, while those of Harrop and Barron were done with phosphate-Ringer.

Influence of phosphate.

In opposition to Dixon and Elliott [1929], Warburg [1931] has strongly emphasised the unphysiological action of phosphates. Using phosphate solution, Dixon found that the inhibition of tissue respiration by cyanide was incomplete; in other words, the phosphate solution caused a relative increase in the respiration. An effect of phosphate in the same sense was apparently observed by Barron, since he reports that in phosphate solution the catalytic action of methylene blue on the respiration of rabbit erythrocytes is increased. Table I shows that in normal rabbit kidney in the presence of phosphates pyocyanine causes a considerable increase in respiration which does not occur in carbonate-Ringer.

In Barron's [1930] experiments methylene blue inhibited the respiration of rat kidney even in a phosphate-Ringer solution, which in itself tends to increase the catalytic action. On the other hand, under the same conditions pyocyanine induces an increase of 16·8 %. Hence the difference in toxicity of the two dyestuffs is readily demonstrated.

Recently Dickens and Šimer [1930] have used phosphate-Ringer for determining the R.Q. in tissue sections. In control experiments these authors have compared the standard values of the respiration ($\bar{Q}_{O_2}$) in carbonate- and in phosphate-Ringer. These authors, having obtained results which coincide within the limits of experimental error, feel justified in assuming the conditions in phosphate-Ringer to be physiological and in according an important rôle to the values of the R.Q. determined in such solutions. In view of the above experiments it seems to us that the comparability of the standard values of respiration in carbonate- and phosphate-Ringer offers no guarantee that the tissues in phosphate-Ringer are under physiological conditions. The great increase in respiration caused by pyocyanine in phosphate- but not in carbonate-Ringer in normal rat kidney seems strongly to indicate that in phosphate solution the oxidation mechanism of the cell is fundamentally different.

The mode of action of pyocyanine.

Increase in depth of respiring layer of tissue sections. Warburg [1923] has drawn attention to the fact that the metabolic exchange is restricted to a more or less thick cortical layer, while the central portion remains inactive. The thickness of

the layer where molecular oxygen is available can be calculated according to Warburg as

$$d = \sqrt{8 c_0 \frac{D}{A}}$$

where d = thickness of layer where oxygen is available,
c_0 = oxygen concentration at the limits between section and solution,
D = diffusion constant of oxygen for tissue substance,
A = the oxygen consumption of the tissue per unit volume and time.

If we regard D as the sum of the diffusion constants of all substances which can act with respect to the tissue as hydrogen acceptors, then D will be increased on addition of pyocyanine by a value equivalent to the diffusion constant of the dye solution in the tissue. As pyocyanine, and to a lesser extent methylene blue, are extremely lipoid-soluble, they have a tendency to accumulate in the tissues to a concentration exceeding the value which would be reached by simple diffusion. Thus there results a considerable increase in D with a corresponding increase in d according to the formula. So far this would only signify an increase in the depth of the tissue layer taking an active part in oxidation metabolism but have no bearing on the measurable oxygen consumption. But as this process goes on, the reduced form of the dye tends to accumulate in the depth of the tissue and a diffusion gradient of the leuco-compound will be established from within the tissue towards the surface (and perhaps oxidisable substances produced by the dehydrogenation of the tissue). Here the leuco-compound, diffusing out of the tissue will immediately be oxidised by the molecular oxygen and thus the oxygen consumption actually increased. It follows that in tissue sections which exceed the thickness in which molecular oxygen would be available, the addition of reducible dyestuffs induces an increase in respiration which is purely mechanical without involvement of the fundamental chemistry of the cell.

Table IV. *Influence of thickness of section on the respiration of rat kidney.*

Ringer solution	2·5/1000 M $NaHCO_3$
Glucose	0·2 %
Final pyocyanine concentration	M/500
Atmosphere	air
Absorption of CO_2 by NaOH	
Temperature	37·6°

	Thickness of section 0·2 mm.			Thickness of section 3·0 mm.		
	mm.^{3} O_2 consumed in 30 mins.			mm.^{3} O_2 consumed in 30 mins.		
Experiment No.	Without pyocyanine	With pyocyanine	Difference %	Without pyocyanine	With pyocyanine	Difference %
1	46	44·5	−3·2	108	132	+13·3
2	18	16·5	−8·3	55	80	+45·5

This effect is illustrated by Table IV. As has been shown in Table I, the respiration of rat kidney cut in sections not thicker than the layer accessible to molecular oxygen (0·2 mm. in an atmosphere of oxygen) is not increased on the addition of pyocyanine; in sections 3·0 mm. thick pyocyanine enhances the respiration as much as 45 %.

The catalytic action. As has been shown in the case of bacteria [Friedheim, 1931] in thin sections of tissue the mechanism of the respiration brought about by pyocyanine may be explained as a true catalysis in that the dye is reduced by

the tissue and reoxidised by the molecular oxygen. The significance of the increase in respiration for the cell may be judged by the influence of pyocyanine on the aerobic glycolysis.

Table V. *Aerobic glycolysis of various tumours, expressed as* $Q^{O_2}_{CO_2}$, i.e. *mm.*^{3} CO_2 *driven out of a bicarbonate solution by lactic acid formed per hour per mg., dry weight of tissue.*

Ringer solution	0·025 *M* $NaHCO_3$
Glucose	0·2 %
Atmosphere	5 % CO_2 in O_2
Temperature	37·5°
Final pyocyanine concentration	*M*/500
Thickness of tissue section 0·2 mm.	

Experiment No.	Tumour	$Q^{O_2}_{CO_2}$ Without pyocyanine	$Q^{O_2}_{CO_2}$ With pyocyanine	Difference %	Without pyocyanine I Respiration Q_{O_2}	Without pyocyanine II Anaerobic glycolysis	Without pyocyanine III U[1]
1	Cori rat sarcoma	20·1	13·1	−34·7	12·5	26·1	+ 1·1
2	Walker rat carcinoma 256	11·4	9·0	−21	6·36	22·1	+ 8·4
3	Walker rat sarcoma S 20	18·5	7·88	−87·6	13·3	68·2	+41·6
4	„	40·4	25·9	−35·8	17·94	62·5	+32·5
5	„	8·51	0·0	−100			
6	Mouse sarcoma	13·0	5·46	−58			
7	„	15·3	6·0	−60·8			
8	Mouse sarcoma S 27	19·9	9·76	−50·9			
9	Human breast carcinoma	5·2	3·4	−34·6			
10	„	6·8	4·4	−22·2			
11	Human spindle-cell sarcoma	5·28	3·1	−41·3			
12	Cori rat sarcoma	13·0	13·0	0·0	−13·9	24·3	− 3·5
13	Walker rat sarcoma	23·7	22·2	0·0	−16·25	28·6	− 4·4

Table V shows that in a series of tumours the aerobic glycolysis is actually decreased on the addition of pyocyanine. The two exceptions in Table VII (Exps. 12 and 13) happen to be tumours with a negative U value[1], *i.e.* tumours in which theoretically the respiration would be sufficient to suppress aerobic glycolysis. Attention has recently been drawn by several authors to the existence of tumours which show at the same time high respiration and low glycolysis. As suggested by Warburg the respiration in tumours may be not only quantitatively insufficient to dominate glycolysis, but as in the case of ethyl carbylamine poisoning, by the coupling of these two processes the mechanism of the Pasteur-Meyerhof reaction might be broken.

[1] U stands for the anaerobic glycolysis *minus* twice the value of the respiration. This concept of U was introduced by Warburg as a measure of the efficiency of respiration since two molecules of oxygen are sufficient to cause the disappearance of one molecule of lactic acid during the course of the Pasteur-Meyerhof reaction. Where $U < 1$, the respiration is "sufficient" and there is no aerobic glycolysis.

Also in our positive experiments in which pyocyanine decreases aerobic glycolysis without eliminating it entirely it must be remembered that the measurements were carried out in Ringer's solution, which is liable in itself to damage the Pasteur-Meyerhof reaction.

In view of the difficulties in interpretation it might seem that the biological significance of the increased respiration brought about experimentally by accessory catalysts, especially in its bearing on the question of malignancy, might best be brought out not with surviving tissue sections but with living cells in animal experiment, or in tissue cultures, as suggested by Barron[1]. For such experiments it is important to have a catalyst available which combines the properties of minimum toxicity and maximum respiratory catalysis. Pyocyanine appears to fulfil these requirements.

Summary.

1. Pyocyanine increases the respiration of tissues with aerobic glycolysis such as testicular tissue and a series of animal and human tumours.
2. The increase in respiration is dependent on the presence of glucose.
3. In this action one must differentiate between an apparent increase in respiration, brought about in thick tissue sections through an increase in the depth of the respiring layer, and a true catalysis.
4. While in carbonate-Ringer the respiration of normal rat kidney is not increased by pyocyanine, a considerable increase is brought about in phosphate-Ringer. In the presence of phosphate the oxidation mechanism of the cell seems to be fundamentally altered.
5. The toxicity of pyocyanine is compared with that of methylene blue. Pyocyanine shows minimum toxicity and enormous action in increasing respiration.

We wish here to express our gratitude to Drs Murphy, Landsteiner, Walker and Klemperer for furnishing animal and human tumour material.

[1] These experiments are under way.

References.

Barron (1930). *J. Exp. Med.* **52**, 447.
Dickens and Simer (1930). *Biochem. J.* **24**, 1301.
Dixon and Elliott (1929). *Biochem. J.* **23**, 812.
Friedheim (1931). *J. Exp. Med.* **54**, 207.
Harrop and Barron (1928). *J. Exp. Med.* **48**, 207.
Michaelis and Salomon (1930). *J. Gen. Physiol.* **13**, 683.
Warburg (1923). *Biochem. Z.* **142**, 317.
—— (1931). *Biochem. Z.* **231**, 793.

XXVI. NATURAL REVERSIBLE OXIDATION-REDUCTION SYSTEMS AS ACCESSORY CATALYSTS IN RESPIRATION: JUGLON AND LAWSON.

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Lawson, the colouring material contained in the leaves of the henna plant (*Lawsonia inermis*), and juglon, the colouring material contained in the husks of walnuts, both form leuco-compounds when exposed to the influence of various reducing agents. The leuco-compounds at once regenerate the pigment in contact with molecular oxygen. Thus they fulfil one of the main conditions which might enable them to play a part in cell-respiration by acting as autoxidisable acceptors of hydrogen.

This paper deals with the effect of juglon and lawson on cell-respiration, the establishment of the oxidation-reduction potentials of both substances and the correlation of the two series of results.

The comparison between the physicochemical and biological properties of the two substances appears all the more attractive in view of the fact that they are isomeric compounds, their formulae being 2-hydroxynaphthoquinone [Tommasi, 1920] for lawson, and 5-hydroxynaphthoquinone for juglon [Bernthsen and Semper, 1885].

A. *Juglon.*

Preparation. Juglon was obtained by extraction of the husks of walnuts by the method of Bernthsen and Semper and also synthetically by oxidation of 1 : 5-dihydroxynaphthalene with chromic-sulphuric acid mixture and subsequent crystallisation from light petroleum. The natural and synthetic products gave identical results in the physicochemical and biological experiments described below.

Potentiometric titration. In order to determine the oxidation-reduction potentials, 10 ml. of $M/1000$ solution in various mixtures of buffer substances were first reduced by hydrogen in the presence of colloidal palladium and subsequently titrated against benzoquinone in a current of purified nitrogen at a temperature of $20° \pm 0{\cdot}1°$. The potentials were read against two polished platinum electrodes, using a saturated half-cell of calomel as standard. The potentials adjusted themselves rapidly and remained stable, and the readings for both electrodes differed by not more than 0·05 mv. during the middle phase of titration.

The potentials thus determined correspond to the formula of Nernst:

$$E = E_0 + \frac{RT}{n} \log \frac{(OX)}{(RED)}.$$

The system juglon-hydrojuglon can therefore be regarded as thermodynamically reversible (see Table I).

Table I. *Juglon.*

$p_H = 5{\cdot}13$ (phosphate) reduced with Pd-H_2 titrated against quinone at $20{\cdot}0° \pm 0{\cdot}05°$.
Potentials calculated according to the formula $E = 0{\cdot}1332 + 0{\cdot}029 \log \frac{\%\text{ oxidation}}{100 - \%\text{ oxidation}}$.
All potentials referred to the standard hydrogen electrode at 20°.

Quinone ml.	Oxidation	Potential		
		Found	Calculated	Discrepancy
0·00	0·0	—	—	—
0·35	15·8	0·1062	0·1121	−0·0059
0·50	22·6	0·1152	0·1177	−0·0025
0·64	28·8	0·1206	0·1218	−0·0012
0·80	36·1	0·1254	0·1260	−0·0006
1·02	45·9	0·1308	0·1311	−0·0003
1·26	56·7	0·1371	0·1366	+0·0005
1·58	71·2	0·1457	0·1446	+0·0011
1·76	79·3	0·1520	0·1501	+0·0019
2·02	91·0	0·1670*	0·1622	+0·0048
2·17	97·6	0·1866*	0·1799	+0·0067
2·22	100·0	0·2672*	—	—

* Potentials drifting.

The determination of n, *the number of electrons which distinguish the reduced from the oxidised form.* According to the chemical constitution of the substance, n must be 2. This statement is corroborated by the oxidation equivalent found by titration, as well as by the slope of the titration curve.

(*a*) Hydrojuglon uses up equimolecular amounts of benzoquinone during its oxidation.

Exp. Juglon mol. wt.: 174.
Standard solution: 43·557 mg. in 50 ml. ethyl alcohol.
1 ml. corresponds to $0{\cdot}5 \cdot 10^{-5}$ *M*.

Benzoquinone mol. wt.: 108.
Titration solution: 44·16 mg. in 100 ml. dist. H_2O.
1 ml. corresponds to $4{\cdot}083 \cdot 10^{-5}$ *M*.

2 ml. of the standard solution of juglon reduced with Pd-H_2 in 8 ml. of acetate buffer solution of $p_H = 4{\cdot}62$, require 2·44 ml. of titrating solution for oxidation in a current of nitrogen (the complete oxidation being indicated by a jump of potential).

$1{\cdot}0 \cdot 10^{-5}$ *M* juglon is equivalent to $\frac{2{\cdot}44 \times 4{\cdot}083}{100} = 0{\cdot}996 \cdot 10^{-5}$ *M* benzoquinone.

(*b*) At acid reactions, the titration curves clearly show a 30 mv. slope, corresponding to $n = 2$. When the p_H reaches 7, the curves become steeper, without reaching however a slope of 60 mv. This irregularity, which points towards a formation of semiquinone or meroquinone, will not be further discussed here, considering the instability of juglon in alkaline media.

The standard potential E_0 (determined in a mixture of equal parts of the oxidised and reduced forms of the substance) is shown in Fig. 1 and Table II as a function of the p_H. According to Clark and Gibbs, the turning-points of the E_0/p_H curve can be interpreted as representing constants of dissociation (see Table V).

B. *Lawson.*

The pure substance was obtained by extraction from henna leaves by the method of Tommasi; the calcium salt was crystallised and the free hydroxyquinone recrystallised from ether. Electrometric titration by means of the method

Table II.

The normal potential of juglon (mixture of equal parts of the oxidised and reduced form) at varying p_H. Potentials referred to the standard hydrogen electrode at 20°.

p_H	Buffer	E_0	Titrated against
1·994	Citrate	+0·311	Quinone
3·263	,,	+0·239	K_3FeCy_6
4·746	Acetate	+0·154	Quinone
5·133	Phosphate I and II	+0·133	,,
5·707	,,	+0·103	,,
6·263	,,	+0·071	,,
6·799	,,	+0·041	,,
7·316	,,	+0·023	,,
8·001	,,	0·000	,,
8·389	,,	−0·023	,,
9·401	II Phosphate and NaOH	−0·071	,,
10·785	,,	−0·131	,,
11·41	,,	−0·160	$K_2S_2O_8$
11·929	,,	−0·175	Quinone
12·10	,,	−0·185	$K_2S_2O_8$

Table III. *Lawson.*

$p_H = 2·24$ (citrate + HCl) reduced with Pd-H_2 titrated against quinone at 20·0° ± 0·05°.

Potentials calculated according to the formula $E = 0·2198 + 0·029 \log \frac{\% \text{ oxidation}}{100 - \% \text{ oxidation}}$.

All potentials referred to the standard hydrogen electrode at 20°.

Quinone ml.	Oxidation %	Potential		
		Found	Calculated	Discrepancy
0·00	0·0	—	—	—
0·14	7·2	0·1828*	0·1870	−0·0042
0·24	12·4	0·1929	0·1947	−0·0018
0·44	22·9	0·2036	0·2040	−0·0004
0·66	33·8	0·2107	0·2108	−0·0001
0·86	44·3	0·2162	0·2163	−0·0001
1·08	55·6	0·2222	0·2221	+0·0001
1·34	69·0	0·2295	0·2293	+0·0002
1·56	80·6	0·2379	0·2372	+0·0007
1·73	89·0	0·2470	0·2455	+0·0015
1·88	96·8	0·2692*	0·2615	—
1·91	98·0	0·2912*	0·2682	—
1·94	100·0	0·3500*	—	—

* Potentials drifting.

described in the case of juglon shows that we are dealing again with a true reversible system. All the titration curves show a slope of 30 mv., corresponding to $n = 2$. An example is recorded in Table III. The dependence of the standard potential E_0 is shown in Table IV and Fig. 1, and the values for the dissociation constants which can be derived from the former are shown in Table V.

C. *A comparison between the various constants of juglon and lawson.*

The 2-hydroxynaphthoquinone, lawson, is more acid and more "negative" than juglon. As compared with juglon the acid character of lawson is enhanced, not only in the phenolic hydroxyl of the oxidised form, but also in the hydroxyl of the reduced form. In the case of lawson, the first dissociation constant of the reduced form is so close to the constant of the oxidised form that the

Table IV.

The normal potential of lawson (mixture of equal parts of the oxidised and reduced form) at varying p_H. Potentials referred to the standard hydrogen electrode at 20°.

p_H	Buffer	E_0	Titrated against
0·360	*N* HCl + *N*/10 KCl	+0·337	K_3FeCy_6
1·322	Citrate	+0·2757	Quinone
1·423	,,	+0·270	,,
2·245	,,	+0·220	,,
2·460	,,	+0·2087	,,
3·248	,,	+0·1635	,,
3·680	,,	+0·1298	,,
3·881	,,	+0·1205	,,
4·280	,,	+0·0862	,,
4·620	Acetate	+0·061	,,
4·945	,,	+0·032	,,
6·194	Phosphate I and II	−0·071	K_3FeCy_6
6·641	,,	−0·1105	Quinone
7·180	,,	−0·154	,,
7·560	,,	−0·186	K_3FeCy_6
8·718	Veronal	−0·281	Quinone
9·180	,,	−0·318	$K_2S_2O_8$
10·075	II Phosphate and NaOH	−0·3648	Quinone
10·510	,,	−0·395	$K_2S_2O_8$
11·34	,,	−0·428	,,
12·23	,,	−0·461	,,

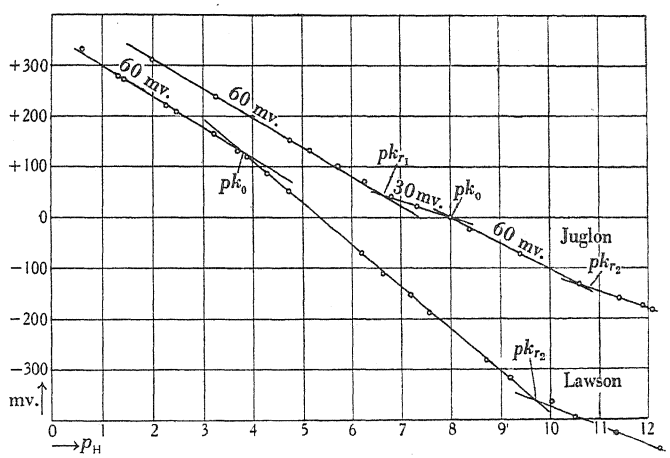

Fig. 1. Normal potentials of juglon and lawson at varied p_H. Potentials referred to the standard hydrogen electrode at 20°.

Table V.

	Juglon	Lawson
Dissociation constant of the oxidised form (of the phenolic hydroxyl)	K 10^{-8}	K $1{\cdot}4.10^{-4}$
1st dissociation constant of the reduced form	$2{\cdot}5.10^{-7}$	About $1{\cdot}4.10^{-4}$
2nd dissociation constant of the reduced form	$2{\cdot}5.10^{-11}$	$2{\cdot}4.10^{-10}$

Temperature: 20°.

two cannot be sharply separated. The flattening effect of the pk_{r_1} combines with the steepening effect of pk_0 to give to the E_0/p_H curve a slope of 84 mv.

Conant and Fieser [1924] have found that the introduction of the hydroxyl group into the quinoid nucleus of α-naphthoquinone lowers the oxidation-reduction potential much more markedly than its introduction into the adjoining benzene ring. Their results support our findings with regard to the increased negativity of lawson as compared with juglon. These authors have determined the oxidation-reduction potentials of the two substances exclusively in strong alcoholic-hydrochloric acid solution. On account of the difference of the solvents the values obtained by them cannot be directly compared with those recorded in this paper.

The relative positions of juglon and lawson in the scale of oxidation-reduction indicators can be seen in Table VI.

Table VI. *Potentials of several oxidation-reduction systems in semi-reduced condition at* $p_H = 7{\cdot}0$.

(Potentials referred to the normal hydrogen electrode.)

Haemoglobin-methaemoglobin	+0·092 ±0·022	Conant und Fieser [1925]
Pigment of *Penicillium phoeniceum*	+0·047	Friedheim [1933, 3]
Juglon	+0·033	
Pigment of *Arion rufus*	+0·025	Friedheim [1933, 1]
Hallachrome	+0·022	Friedheim [1933, 2]
Methylene blue	+0·011	
Pyocyanine	−0·033	Friedheim and Michaelis [1931]
Indigotetrasulphonate	−0·046	Clark, cited from Michaelis [1933]
Indigodisulphonate	−0·125	
Lawson	−0·139	
Echinochrome	−0·220	Cannan [1927]
β-Anthraquinonsulphonate	−0·260	

D. *Juglon and lawson as natural oxidation-reduction indicators.*

In the husks of walnuts, juglon is generally found in the reduced form, lawson on the other hand exists in henna leaves in the oxidised state. If we consider the two pigments as natural intracellular oxidation-reduction indicators, we can infer that the oxidation-reduction potential of the plant-cells under consideration is more negative than the standard potential of juglon and more positive than the standard potential of lawson.

At $p_H = 7{\cdot}0$ this statement may be expressed as:

$$\underset{\text{juglon}}{+0{\cdot}033} > \underset{\text{cell}}{E_h} > \underset{\text{lawson}}{-0{\cdot}139}$$

or in terms of r_H

$$15{\cdot}1 > r_H \text{ cell} > 9{\cdot}2^1.$$

[1] Haas and Hill [1925] have described a plant pigment, hermidin, which has been shown by Cannan [1926] to behave like a reversible oxidation-reduction system. Calculated from the data given by Cannan, the standard potential would assume at p_H 7 a value of E_0 −0·033 and r_H 12·7. These figures fall well within the limits given above. It may be noted that the standard potential of hermidin coincides exactly with that of pyocyanine, the blue pigment of *Bacillus pyocyaneus*.

These limits, although far apart, are important because they hold good under physiological conditions, *e.g.* in air. They refer to the actual equilibrium between processes of oxidation and reduction in the living cell. This equilibrium is the result not only of thermodynamic but also of kinetic conditions, *i.e.* of diffusion and reaction velocities.

We owe our present knowledge of intracellular oxidation-reduction potentials up to date to the works of Needham and Needham [1925], Cohen *et al.* [1928], Wurmser and Rapkin [1931], *etc.* who have used the method of direct introduction of indicators into the living cell. It is important to supplement this method by means of direct observation of natural intracellular oxidation-reduction indicators, as the latter method eliminates possible sources of error such as toxicity of the indicator, trauma of injection *etc.*[1]

E. *The influence of juglon and lawson on the respiration of red corpuscles (rabbits).*

The work of Barron and Harrop [1928], as well as that of Michaelis and Salomon [1929–30], goes to prove that reversible oxidation-reduction systems are capable of increasing the respiration of red corpuscles, if non-toxic, able to permeate and possessing a normal potential more positive than that of indigo-disulphonate. Juglon and lawson, which fulfil these conditions, both behave in accordance with the above-mentioned rule: under their influence the respiration of the erythrocytes of rabbits is several times increased (see Fig. 2).

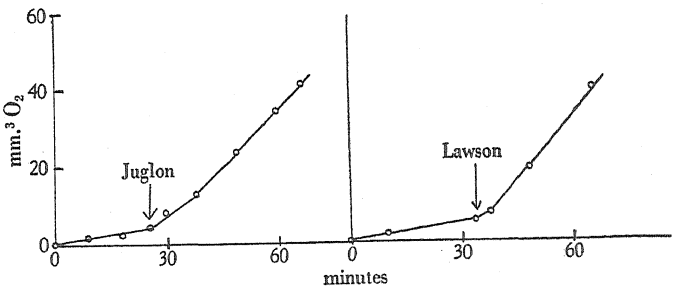

Fig. 2. The action of juglon and lawson on the respiration of red blood cells (rabbit).

Phosphate-Ringer p_H 7·2.
Phosphate concentration $M/75$.
0·2 % glucose.
Absorption of CO_2 by 5 % NaOH.
37° air.

At ↓ the dye is added from the side-bulb. Final dye concentration $M/1000$.

In our experiments juglon increases the respiration 5 times, and lawson 5·8 times. The effect of both these isomeric bodies is of the same numerical order, which however is difficult to reproduce exactly. It is independent of the nature of the medium (0·9 % NaCl, carbonate- or phosphate-Ringer) provided glucose is present.

[1] The conception of "intracellular" is as undefined as an address "London". In the various regions of the cell can be found different oxidation-reduction potentials (*e.g.* granula of oxidase and the "Sauerstofforte" of *Unna*). As fresh material is not available at present a more precise localisation of juglon and lawson will be attempted later on.

F. *The mechanism of increase of respiration in red corpuscles by reversible oxidation-reduction systems.*

Warburg *et al.* [1930] and Wendel [1929–30] have discovered the following mechanism for the increase of respiration of red corpuscles in the presence of methylene blue:

1. Methylene blue + haemoglobin + H_2O = methaemoglobin + leuco-methylene blue.

2. Methaemoglobin + carbohydrate = haemoglobin + oxidation products of carbohydrate.

3. Leuco-methylene blue + 1/2 O_2 = methylene blue + H_2O.

Michaelis and Salomon [1929–30] have further confirmed this theory for a number of reversible dyes and have brought it into correlation with the normal potentials of these dyes. The potential interval between indigodisulphonate and indigotetrasulphonate was defined as the critical level, on the positive side of which the increase in respiration runs approximately parallel with the formation of methaemoglobin. On the negative side, dyes are known to exist which can form methaemoglobin (indigodisulphonate, β-anthraquinonesulphonate, safranine) without exercising any influence on the respiration. This latter circumstance strengthens the view according to which the formation of methaemoglobin need not necessarily represent the crucial process in the increase of respiration by quinoid dyes.

Let us consider the conditions prevailing in the case of our hydroxynaphthoquinones: lawson and juglon both increase the respiration of the red corpuscles to considerable and approximately equal extents. But whereas the positive juglon, the normal potential of which is almost identical with that of methylene blue, rapidly oxidises haemoglobin to methaemoglobin, lawson, which is only a few mv. more negative than indigodisulphonate (at p_H 7) forms no methaemoglobin under conditions where an increase of respiration takes place. This has been ascertained from spectroscopic observation as well as from the determination of the oxygen capacity.

Table VII. *Oxygen capacity of one ml. of a suspension of red blood corpuscles (rabbits)**.

Temperature		mm.^{3} O_2
I. 25°	Control	61·5
	40 mins. after addition of M/1000 lawson (duplicate experiment)	(*a*) 61·7
	Spectroscopically: no methaemoglobin	(*b*) 62·0
II. 37°	Control	91·9
	40 mins. after addition of M/1000 lawson Spectroscopically: no methaemoglobin	91·6
	40 mins. after addition of M/1000 juglon Typical methaemoglobin bands at 635, shifted towards 610 under the influence of NaF	25·5 =28 %

* Method of Warburg *et al.* [1931].

Certain quinoid dyestuffs produce methaemoglobin without provoking any increase of respiration, whereas others, such as lawson, increase respiration without producing methaemoglobin. Thus the catalytic action of quinoid dyes on the respiration of red corpuscles is not indissolubly linked up with the action of the haemoglobin-iron.

The limits of potential of the juglon-hydrojuglon system overlap widely with those of the haemoglobin-methaemoglobin system. It is therefore possible for juglon to oxidise haemoglobin. At p_H 7·0 juglon, oxidised to approx. 99 % ($E_h = +0{\cdot}093$), forms a thermodynamical equilibrium with haemoglobin oxidised to about 50 % ($E_h = +0{\cdot}092 \pm 0{\cdot}022$). On the other hand, lawson, oxidised to the extent of 99 % ($E_h = -0{\cdot}079$) does not come within the range of the potential of a haemoglobin-methaemoglobin system reduced to the extent of 99 % ($E_h = -0{\cdot}028 \pm 0{\cdot}022$). The formation of methaemoglobin in the presence of lawson appears therefore rather improbable.

With regard to the formation of methaemoglobin by means of quinoid dyes, one should remember that the thermodynamical possibility, as expressed in terms of the standard potentials, is no guarantee that the reaction actually takes place.

β-Anthraquinonesulphonate oxidises haemoglobin: the more positive lawson ought to have at least a similar effect. But "kinetic factors outweigh thermodynamic factors in all reactions concerning haemoglobin" (Michaelis). Even after ten hours of exposure of red corpuscles to the action of lawson, no formation of methaemoglobin could be observed. Neither would a belated appearance of methaemoglobin be of any significance with regard to the mechanism of increase of respiration, as our experiments have shown that the latter itself appears immediately when lawson has been brought into contact with red corpuscles[1].

[1] I have described a number of natural reversible oxidation-reduction systems as "accessory respiratory enzymes." Stern [1933] questions the advisability of applying the term "enzyme" to the cases where methaemoglobin appears during the reaction. Whether or not these systems should be called enzymes depends entirely on the accepted definition.

Kuhn's definition in the work on ferments by Oppenheimer that "ferments are catalysts manufactured by the cell," holds good for the chemical bodies with which we are concerned, including juglon and lawson. If one accepts on the other hand Haldane's restriction of the definition to the effect that "ferments are colloidal catalysts," then these crystalline substances cannot be regarded as ferments.

By no means can we consider the nature of intermediate products, *e.g.* the possible formation of methaemoglobin, as an argument either for or against the enzymic character of the reaction.

As in this paper the formation of methaemoglobin has been shown to be irrelevant for the enhancing effect of reversible oxidation-reduction systems on respiration of red blood cells, Stern's discussion appears all the more beside the point.

REFERENCES.

Barron and Harrop (1928). *J. Biol. Chem.* **79**, 65.
Bernthsen and Semper (1885). *Ber. deutsch. chem. Ges.* **18**, 203.
Cannan (1926). *Biochem. J.* **20**, 927.
—— (1927). *Biochem. J.* **21**, 184.
Cohen, Chambers and Retznikoff (1928). *J. Gen. Physiol.* **11**, 585.
Conant and Fieser (1924). *J. Amer. Chem. Soc.* **46**, 1858.
—— —— (1925). *J. Biol. Chem.* **62**, 595.
Friedheim (1933, 1). *Compt. Rend. Soc. Phys. Hist. Nat. Geneva*, **50**, 162.
—— (1933, 2). *Biochem. Z.* **259**, 257.
—— (1933, 3). *Compt. Rend. Soc. Biol.* **112**, 1030.
—— and Michaelis (1931). *J. Biol. Chem.* **91**, 355.

Haas and Hill (1925). *Biochem. J.* **19**, 233.
Michaelis (1933). Oxydation-Reduktion Potentiale, 103. (Springer, Berlin.)
—— and Salomon (1929–30). *J. Gen. Physiol.* **13**, 683.
Needham and Needham (1925). *Proc. Roy. Soc. Lond.* B **98**, 259.
Stern (1933). *Naturwiss.* **21**, 350.
Tommasi (1920). *Gazz. Chim.* **50**, 263.
Warburg, Kubowitz and Christian (1930). *Biochem. Z.* **227**, 245.
—— —— —— (1931). *Biochem. Z.* **242**, 173.
Wendel (1929–30). *Proc. Soc. Exp. Biol. Med.* **27**, 624.
Wurmser and Rapkin (1931). *Compt. Rend. Acad. Sci.* **193**, 430.

XXVII. A NOTE ON THE PRESENCE OF VITAMIN C IN THE CHICK EMBRYO.

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It has long been known that fowls and other birds resemble rats [Parsons, 1920] and dogs [Harris, 1931] in having little apparent need for vitamin C in their diet. Plimmer *et al.* [1923] concluded that the growth of the chick in the egg, which is known to be free from vitamin C, is one of the best indications that vitamin C is not needed by the growing bird. This statement has been criticised on the ground that the avian embryos may synthesise the vitamin during their development, and indeed Carrick and Hauge [1925] and Hart *et al.* [1925] demonstrated the presence of vitamin C in the livers of chicks which had been fed for a long time on vitamin C-free diets. Hauge and Carrick [1926] further showed that though both the yolks and the whites of eggs laid by hens which had been on scorbutic ration contained no demonstrable amount of vitamin C, yet the livers of five-weeks-old chicks hatched from these eggs and fed on scorbutic rations contained an abundance of this vitamin. This makes it evident that during incubation vitamin C is being produced in the growing embryo.

The earlier studies, which involved the employment of large amounts of embryonic tissue for biological assay, prevented the actual detection of vitamin C in the growing embryo. The much simpler micro-chemical method of Birch *et al.* [1933] makes the demonstration of the vitamin an easy and rapid affair.

Experimental.

Brown Leghorn hens' eggs were used. The eggs after the proper time of incubation were opened, the embryos removed and dried on filter-paper. They were rapidly weighed and ground up with a little sand and sufficient 20 % solution of trichloroacetic acid to give a final concentration of the latter of about

Table I.

Day of incubation	Weight of the substance taken g.		Total amount of ascorbic acid mg.	Amount of ascorbic acid in mg./g. wet tissue	Amount of ascorbic acid in mg./g. dry tissue
0	(*a*) Egg-white	27	0	0	0
	(*b*) Egg-yolk	20	0	0	0
4	(*a*) 5 whole embryos	0·6	0·0745	0·124	2·98
	(*b*) Egg-white	11·6	0	0	0
	(*c*) Egg-yolk	20	0	0	0
6	2 whole embryos	0·89	0·143	0·160	3·04
8	1 ,,	1·17	0·213	0·182	2·91
10	1 ,,	2·68	0·630	0·235	3·38
12	1 ,,	5·05	1·260	0·249	2·81
15	Embryo livers	0·50	0·132	0·264	—
17	,,	0·37	0·105	0·283	—
19	,,	0·65	0·178	0·274	—
21	,,	0·82	0·226	0·275	—
24 hours old chick-liver		1·05	0·273	0·260	—

5 %. The extract was then made up to a suitable volume and the filtrate titrated against a measured volume of a standard solution of 2 : 6-dichlorophenolindophenol. Up to the 15th day the whole embryos were used; on the 15th day and after, the livers only were examined.

The dry weight of the embryo, used to calculate the figures in the last column of the table, was obtained from Murray's [1926] figures for chick embryo. As the wet weights were found to be nearly identical with his values of wet weight of the embryo, it may be taken that the figures in the last column are reasonably accurate.

Discussion.

It is evident from Table I that while there is no reducing substance in the fresh hens' eggs, quite large amounts of such substances appear in the embryo on incubation. It is also known from previous work [Birch *et al.* 1933; Harris, 1933] that only a few substances other than ascorbic acid reduce the dye under the conditions of the experiment. As these other substances are not likely to be present in the fresh embryo tissue, the amount of reduction can be taken to be a fair indication of the amount of vitamin C present. Further, Hart *et al.* [1925] found that 3 g. of liver from a chicken kept on scorbutic ration for 83 days, constitute a curative dose for scorbutic guinea-pigs; while 1 g. of such liver is definitely not sufficient. If we now take 0·9 mg. of ascorbic acid as the minimum curative dose [Harris and Ray, 1933, 1, 2] and take from Table I the average amount present in liver to be 0·26 mg., the minimum curative dose for chicken liver works out to be 3·4 g. This value is in close agreement with the figures given by Hart *et al.* [1925].

The appearance of vitamin C with the commencement of growth is also seen in the germination of seeds. Moreover, it may be seen from Table I that the amount of vitamin C present per g. of the dry embryo tissue is fairly constant. These facts naturally suggest that there may be some connection between the two phenomena—the appearance of vitamin C and the beginning of growth. In order to determine whether excess of vitamin C has any influence on the rate of growth, some preliminary experiments were performed by injecting about 2 mg. of ascorbic acid (ten times the amount present in the developing embryo) in sterile aqueous solution into the air-space of the eggs before incubation. The result was however totally negative. From both the untreated and treated eggs, the chickens came out simultaneously after the usual incubation period of 21 days, nor was there any great difference between the weights of the two classes. It does not seem likely therefore that moderate excesses of vitamin C have any further direct action on the growth.

Summary.

1. The embryo of the chick has been examined for vitamin C. There is no vitamin present in the fresh egg, though quite a large amount is present even after only 4 days' incubation. The amount of vitamin C present in embryo chick liver agrees very well with the value given by Hart *et al.* [1925] for livers of chickens kept for a long time on a scorbutic ration.

2. Aqueous solutions of vitamin C when injected into the hen's egg do not seem to produce any change in the growth-rate of the embryo.

My thanks are due to Sir F. G. Hopkins for his kind interest in this work. I am indebted also to Dr L. J. Harris and Dr J. Needham for their constant advice and encouragement.

REFERENCES.

Birch, Harris and Ray (1933). *Biochem. J.* **27**, 590.
Carrick and Hauge (1925). *J. Biol. Chem.* **63**, 115.
Harris (1931). Quoted by Innes, *Ann. Rep. Inst. Animal Path.* Cambridge (1931), p. 150.
—— (1933). *Nature*, **132**, 27.
Harris and Ray (1933,1). *Biochem. J.* **27**, 580.
—— —— (1933, 2). *Biochem. J.* **27**, 2016.
Hart, Steenbock, Lepkovsky and Halpin (1925). *J. Biol. Chem.* **66**, 813.
Hauge and Carrick (1926). *Poultry Sci.* **5**, 166.
Murray (1926). *J. Gen. Physiol.* **9**, 39.
Parsons (1920). *J. Biol. Chem.* **44**, 587.
Plimmer, Rosedale and Raymond (1923). *Biochem. J.* **17**, 787.

XXVIII. THE ENZYMIC FORMATION OF HYDROGEN SULPHIDE BY CERTAIN HETEROTROPHIC BACTERIA. II.

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(*Received January 1st, 1934.*)

In a previous communication [Tarr, 1933] the formation of hydrogen sulphide from organic sulphur compounds by certain heterotrophic bacteria was attributed to the activity of a specific enzyme present in their cells. Evidence was also obtained in support of the fact that different mechanisms are responsible for the formation of hydrogen sulphide from organic sulphur compounds and from thiosulphate by such bacteria. This evidence has been substantiated by the results obtained in an experiment described in the present paper. It is possible that the formation of hydrogen sulphide from inorganic sulphur compounds is also due to different mechanisms. Thus the sulphate bacteria produce hydrogen sulphide from sulphate, sulphite and thiosulphate [Stephenson and Stickland, 1931], and from hyposulphite and sulphur as well [Baars, 1930], while *P. vulgaris* cells only reduce thiosulphate and sulphur to hydrogen sulphide [Tarr, 1933]. The experimental work described in this paper has been almost entirely confined to the formation of hydrogen sulphide from organic compounds.

Experimental.

The technique employed was essentially the same as that previously described [Tarr, 1933]. Unless otherwise stated all experiments were made anaerobically in evacuated 75 ml. Thunberg tubes, each tube containing 5 ml. of bacterial suspension, 2 ml. of $M/5$ phosphate buffer, p_H 7·8, the required amount of substrate solution and a final fluid volume of 20 ml. Strictly aseptic precautions were only observed in experiments the duration of which was over 2 hours.

The homocystine used was kindly given to me by Prof. V. du Vigneaud, the dithiodihydracrylic acid (disulphide from β-thiolpropionic acid) by Dr A. Schöberl, and the *dl*-cysteine hydrochloride by Dr N. W. Pirie. Solutions of these compounds were prepared in accordance with the general procedure previously described [Tarr, 1933].

The formation of hydrogen sulphide from cysteine and from thiosulphate by P. vulgaris *cells.*

It was previously shown [Tarr, 1933] that hydrogen sulphide is formed from cysteine by cells of both *P. vulgaris* and *S. marcescens*, but from thiosulphate only by the former organism, thus indicating that two different systems capable

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of forming hydrogen sulphide from these substrates exist. The following experiment shows that *P. vulgaris* cells possess both systems.

Ten Thunberg tubes containing buffer and *P. vulgaris* cells as usual were prepared. One pair of tubes received 5 mg. of $Na_2S_2O_3$ per tube, a second pair 6 mg. of $Na_2S_2O_3$, a third pair 1 mg. of cysteine, and a fourth pair 5 mg. of $Na_2S_2O_3$ *plus* 1 mg. of cysteine. A fifth pair was retained as control. The tubes were evacuated as usual and placed in a water-bath at 37° for 3½ hours, and the amount of hydrogen sulphide formed was then determined. The results of this experiment, which are given in Table I, show that the addition of cysteine to

Table I. *The effect of the addition of cysteine on the production of H_2S by* P. vulgaris *cells saturated with respect to $Na_2S_2O_3$.*

Experiment	H_2S in γ after 3½ hours at 37°	
	Duplicates	Average
5 mg. $Na_2S_2O_3$	67 69	68
6 mg. $Na_2S_2O_3$	70 74	72
1 mg. cysteine	67 65	66
1 mg. cysteine + 5 mg. $Na_2S_2O_3$	126 126	126

23 mg. dry weight of bacteria per exp. Controls formed no H_2S.

P. vulgaris cells which are saturated with respect to thiosulphate results in an increase in hydrogen sulphide formation almost equivalent (91 %) to the sum of the hydrogen sulphide formed from cysteine and thiosulphate alone. This may be considered practically conclusive proof that H_2S formation from thiosulphate and from cysteine by *P. vulgaris* is due to two entirely different mechanisms.

The action of P. vulgaris *and* S. marcescens *cells on* l-*cysteine,* dl-*cysteine, homocystine and the disulphide from β-thiolpropionic acid.*

The technique employed was identical with that previously used in studying H_2S formation from different compounds [Tarr, 1933], and the results of the experiment are given in Table II. The action on *l*-cysteine was studied in order that the results obtained might be strictly comparable with those recorded in the previous paper. Although the disulphide from β-thiolpropionic acid is reduced to the —SH form by both organisms, as is evidenced by a strong nitroprusside reaction, neither of them forms H_2S from it. On the other hand homocystine is reduced by both bacteria, with simultaneous formation of H_2S in small amounts. *S. marcescens* forms H_2S from *l*-cysteine and from *dl*-cysteine equally well, but *P. vulgaris* exhibits greater activity in this respect toward the former compound. The facts that most of the sulphur of cysteine can be recovered as H_2S, while none of the sulphur of the corresponding acid which lacks an amino-group yields H_2S, make it appear highly probable that the presence of an amino-group, and consequently deamination, is necessary to the successful course of the reaction. Thus homocystine which contains an amino-group yields H_2S under the experimental conditions in spite of the fact that the —SH group is on the γ-carbon atom.

Table II. *The anaerobic formation of H_2S from* l-*cysteine,* dl-*cysteine, homocystine and the disulphide from β-thiolpropionic acid by washed cells of* P. vulgaris *and* S. marcescens.

Compound	Weight used mg.	Theoretical amount of H_2S in mg.	H_2S recovered: *P. vulgaris* mg.	*P. vulgaris* % *	*S. marcescens* mg.	*S. marcescens* % *
l-Cysteine	0·5	0·141	0·120 0·115	79	0·120 0·112	79
dl-Cysteine	0·5	0·141	0·073 0·075	49	0·115 0·120	80
Homocystine	0·5	0·126	0·033 0·038	23	0·018 0·020	12
Disulphide from β-thiolpropionic acid	0·5	0·162	0·006 0·006	0	0·004 0·004	0
Controls	0·0	0·0	0·006 0·006	0	0·004 0·004	0

* Calculated on the theoretical value after subtracting the control.

P. vulgaris 28 mg. dry weight of bacteria per exp.
S. marcescens 54 ,, ,,

The extraction from P. vulgaris *cells of a cell-free preparation of the enzyme forming H_2S from cysteine.*

The procedure to be described was conducted under as nearly aseptic conditions as possible in order to avoid the presence of contaminating bacteria in the final preparations. The suspensions of *P. vulgaris* cells were prepared as usual, the cells being suspended in distilled water instead of Ringer solution, so that a suspension containing about 50 mg. dry weight of bacteria in 10 ml. was obtained. Suspensions prepared in this manner were run slowly, and with rapid stirring, into 10 volumes of acetone. The resulting flocculent precipitate was permitted to settle and the supernatant liquid carefully syphoned off, and a second 10 volumes of acetone were added. The precipitate was then collected on a small Büchner funnel, pressed between sterile filter-papers and dried for 2–3 hours at 28°. The resulting dry brittle mass was used within 2 days of preparation.

The acetone-dried bacteria were ground to a fine powder in a small mortar and mixed with *M*/20 phosphate buffer (p_H 7·8), 10 ml. being used for each 100 mg. of bacteria. The resulting suspension (A) was incubated for 2 hours at 37° and then centrifuged for 1 hour at high speed. The faintly opalescent, pale yellow supernatant liquid (B) was decanted, and the precipitate (C) was resuspended in distilled water and made up to a volume equivalent to that of the original suspension. Some of liquid B was filtered through a layer of kieselguhr, and some through a Seitz asbestos filter, and the resulting almost water-clear solutions labelled (D) and (E) respectively. A portion of the kieselguhr filtrate was heated to 80° for 2 minutes (F). The ability of these various fractions to form H_2S from 10 mg. of cysteine was determined, corresponding controls without cysteine being run in each case. The results, which are given in Table III, show that suspensions of acetone-treated bacteria form small amounts of H_2S, though calculation has shown that such preparations possess only 2–3 % of the original activity of the freshly prepared cells. On centrifuging suspensions of acetone-bacteria about one-half of the active principle remains in the supernatant liquid, while the remaining half is to be

found in the precipitate. Filtering the supernatant liquid through kieselguhr yields a clear, cell-free fluid, which retains the activity of the original liquid in full. On the other hand all the activity is lost by filtering the solution through a Seitz filter. Heating to 80° for 2 minutes renders kieselguhr filtrates inactive.

Table III. *The formation of H_2S by extracts of acetone-dried* P. vulgaris *cells.*

Preparation (10 ml. per exp.)	H_2S in γ produced from 10 mg. of cysteine in 4 hours at 37°
A	45
B	20
C	23
D	19
E	0
F	0

Controls gave no H_2S. Bacterial examinations showed that all solutions prepared under such conditions are sterile or nearly so.

All attempts to obtain active cell-free enzyme preparations by autolysing *P. vulgaris* cells for a week at 37° in phosphate buffer of p_H 7·8 or by drying cells *in vacuo* and extracting them with phosphate buffer have proved unsuccessful.

The properties of the enzyme forming H_2S from cysteine as determined by the use of intact P. vulgaris *cells.*

The effect of p_H on the velocity of H_2S formation from cysteine was determined. Phthalate-NaOH and KH_2PO_4-NaOH buffers (Clark and Lubs), and a glycine-NaCl-NaOH buffer (Sørensen) were employed. (Unpublished experiments have shown that *P. vulgaris* cells do not decompose glycine under anaerobic conditions.) The amount of H_2S formed from 1 mg. of cysteine in 4 hours at 37° was estimated at various p_H values, 2 ml. of the required buffer solution being employed in each experiment. The H_2S formed in the absence of cysteine under identical conditions was subtracted in each case. The results of this experiment are shown in Fig. 1. A study of this figure indicates that the

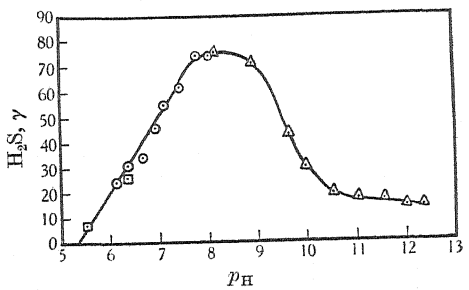

Fig. 1. The p_H-activity curve of the enzyme forming H_2S from cysteine.
⊡ Phthalate-NaOH buffer. 34 mg. dry weight of bacteria per experiment.
⊙ KH_2PO_4-NaOH buffer.
△ Glycine-NaCl-NaOH buffer.

reaction proceeds with maximum velocity between p_H 7·8 and 9·0, there being a rapid decline in activity at higher and lower p_H values respectively. The enzyme is inactive at p_H 5·5. Curiously enough the enzyme is still active at p_H 12, although no H_2S is obtained from cysteine alone at this p_H under the

experimental conditions. Additional experiments have shown that if washed *P. vulgaris* cells are held at 37° for 5 minutes in *M*/20 glycine-NaCl-NaOH buffer of p_H 12 and the p_H then adjusted to approximately 7·8, about 37 % of the original activity of the cells remains. Thus in one experiment untreated cells formed 80γ of H_2S from 10 mg. of cysteine in 1 hour at 37°, and cells treated as above described formed 30γ of H_2S under similar conditions.

In Fig. 2 the effect of temperature on the amount of H_2S formed from 1 mg. of cysteine in 4 hours is shown. It is evident from this figure that the reaction has a relatively sharp optimum temperature of about 40°, and that an extremely rapid decline in activity is exhibited at higher or lower temperatures. The fact that the enzyme is inactive at 50° indicates that it is fairly sensitive to heat.

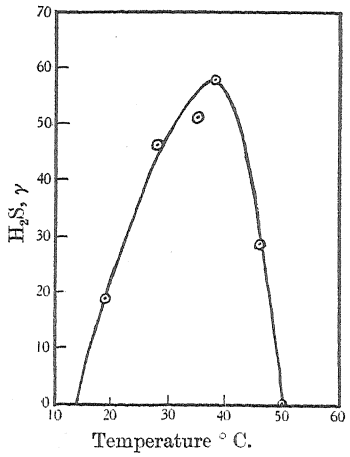

Fig. 2. The effect of temperature on the velocity of H_2S formation from cysteine by *P. vulgaris* cells.

26 mg. dry weight of bacteria per experiment.

The influence of aerobic and anaerobic conditions on the velocity of H_2S formation from 10 mg. of cysteine in ½ hour at 37° was determined. In one experiment the Thunberg tubes were evacuated in the usual manner, in a second a rapid stream of nitrogen washed thoroughly through alkaline pyrogallol solution was bubbled through the solutions during the course of the reaction, and in a third a stream of air was used in place of nitrogen. In the last two of these experiments the H_2S formed was absorbed in zinc acetate solution during the course of the reaction. In all experiments after half an hour the experimental solution was acidified with H_2SO_4 in the usual manner, and the H_2S present aerated into the zinc acetate solution. The results of these experiments, recorded in Table IV, indicate that H_2S is formed much more rapidly from cysteine under aerobic than under anaerobic conditions, this result being in agreement with the data obtained by Almy and James [1926] for *P. vulgaris* grown in a cystine-containing medium. In addition it is evident from Table IV that the velocity of H_2S formation is greater under the anaerobic

Table IV. *The effect of aerobic and anaerobic conditions on the formation of H_2S from cysteine by* P. vulgaris.

Conditions of experiment	H_2S in γ after ½ hour at 37°	
	Duplicates	Average
Aeration with air	141 141	141
Nitrogen passed through the solutions	80 80	80
Evacuated tubes	60 58	59

Controls without cysteine gave no H_2S. 22 mg. of dry weight of bacteria per exp.

conditions created by passing a stream of nitrogen through the experimental liquid than under similar conditions created by a partial vacuum. It is possible that this last-named effect is due to the fact that in experiments in which

nitrogen is employed the reaction product (H_2S) is more rapidly removed from the seat of the reaction than in experiments *in vacuo*.

The influence of cyanide on the velocity of H_2S formation was studied, 10 mg. of cysteine being employed in each experiment. All experiments were carried out at 37°, H_2S being estimated after $\frac{1}{2}$ and 1 hour. 5 ml. of $M/5$ phosphate buffer of p_H 7·8 were used instead of the usual 2 ml. The effect of cyanide was investigated by placing a small tube containing 1 ml. of KCN solution of appropriate concentration in the fluid in the Thunberg tube employed and mixing the liquids subsequent to evacuation. It is evident from the results given in Table V that KCN in the concentrations employed causes only a partial inhibition of the reaction. The inhibition due to a given cyanide concentration decreases with time, and the reaction is, therefore, reversible.

Table V. *The effect of KCN on the velocity of H_2S formation from cysteine by* P. vulgaris.

	H_2S formed in γ from 10 mg. cysteine			
Experiment	$\frac{1}{2}$ hour	% inhibition	1 hour	% inhibition
Controls (no KCN)	84	—	120	—
	84		126	
$M/1000$ KCN	60	29	100	20
	60		95	
$M/500$ KCN	36	56	76	37
	38		78	

24 mg. dry weight of bacteria per exp. Controls without cysteine gave no H_2S.

A study was also made of the effect of toluene treatment, 5 % ethylurethane and phenylurethane (saturated solution) on the velocity of H_2S formation from cysteine. The toluene-treated bacteria were prepared according to the method of Quastel and Wooldridge [1928], and all attempts to obtain growth of bacteria from such suspensions on caseinogen digest agar were unsuccessful. The results of these experiments (Table VI) show that phenylurethane causes no inhibition

Table VI. *The effect of toluene treatment, phenylurethane and ethylurethane on the velocity of H_2S formation from cysteine by* P. vulgaris.

Experiment	H_2S in γ from 10 mg. cysteine in 1 hour at 37°	% inhibition
Controls	97	0
	97	
Toluene-treated cells	54	45
	54	
5 % ethylurethane	36	62
	38	
Saturated phenylurethane	95	0
	100	

34 mg. dry weight of bacteria per exp. Control exps. without cysteine gave no H_2S.

of the reaction, while 5 % ethylurethane inhibits the reaction by about 60 %. It is interesting to note that toluene-treated *P. vulgaris* cells, which are "non-viable" as far as ability to reproduce on culture media is concerned, still retain over 50 % of their ability to form H_2S.

SUMMARY.

1. Hydrogen sulphide formation from cysteine and from thiosulphate by washed *P. vulgaris* cells is due to two different mechanisms. The formation of H_2S from organic sulphur compounds by this bacterium is due to the activity of a specific enzyme present in its cells.

2. Sterile, cell-free extracts containing small amounts of this enzyme can be obtained by extracting acetone-dried *P. vulgaris* cells with phosphate buffer, centrifuging and filtering the resulting supernatant liquid through kieselguhr. Such extracts exhibit only 2–3 % of the original activity of the cells from which they are prepared.

3. This enzyme as it exists in intact *P. vulgaris* and *S. marcescens* cells apparently requires the presence of an amino-group before it can become active. Thus H_2S is formed from α-amino-β-thiolpropionic acid (cysteine) but not from β-thiolpropionic acid, while α-amino-γ-thiolbutyric acid (homocysteine) gives rise to small amounts of H_2S.

4. The enzyme as it exists in intact *P. vulgaris* cells is inactive at p_H 5·5, is most active at p_H 7·8–9·0, and retains nearly 20 % of its optimum activity at p_H 12. It has an optimum temperature of 40° and is inactive at 50°. More H_2S is formed in a given time by the enzyme under aerobic than under anaerobic conditions. Non-viable, toluene-treated bacteria retain over 50 % of their activity with respect to H_2S formation. Phenylurethane does not inhibit H_2S formation from cysteine, while 5 % ethylurethane causes some 60 % inhibition of this reaction. Cyanide only partially inhibits the reaction, and this inhibition is reversible.

I am greatly indebted to Prof. V. du Vigneaud of George Washington University for his kindness in giving me the homocystine used in this work, to Dr A. Schöberl of the Chemisches Inst. der Univ. Würzburg for a supply of dithiodihydracrylic acid, and to Dr Pirie of this Department for the *dl*-cysteine hydrochloride employed. For his constant interest and encouragement I wish to thank Sir F. G. Hopkins.

REFERENCES.

Almy and James (1926). *J. Bact.* **12**, 319.
Baars (1930). Over het sulphaat reductie door bacterie. (Dissertation, Delft.)
Quastel and Wooldridge (1928). *Biochem. J.* **22**, 689.
Stephenson and Stickland (1931). *Biochem. J.* **25**, 215.
Tarr (1933). *Biochem. J.* **27**, 1869.

XXIX. THE DETERMINATION OF THE MEMBRANE POTENTIALS OF PROTEIN SOLUTIONS AND THE VALENCE OF PROTEIN IONS.

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1. *Introduction.*

The theoretical treatment of membrane potentials in very dilute solutions is mainly due to Donnan [1911], who considered a system in which a solution containing the sodium salt of a colloid such as Congo red is separated from a solution of sodium chloride by a membrane permeable by water and crystalloids but impermeable by the colloidal particles. After equilibrium has been reached, the concentration of sodium ions is greater in the solution inside the membrane, containing the colloid, and there is a potential difference between the colloidal solution and the salt solution or dialysate, determined by Nernst's formula

$$\frac{E}{1000} = \frac{-RT}{F} . ln \frac{[Na]'}{[Na]''} = \frac{-2{\cdot}303RT}{F} \log \frac{[Na]'}{[Na]''} \qquad \ldots\ldots(1).$$

E = the potential difference or membrane potential in millivolts. $F = 96{,}494$ coulombs. $R = 8{\cdot}316$ volt coulombs. T = the absolute temperature. $[Na]'$ = the concentration of sodium ions in the colloidal solution. $[Na]''$ = the concentration of sodium ions in the dialysate.

The sign of the potential is the same as the sign of the colloidal particle, negative in the case of Congo red and positive in the solutions of protein chlorides studied by Loeb [1922], who devised a method for the experimental investigation of membrane potentials.

An extension of the theory to more concentrated solutions was made by Donnan and Allmand [1914], who derived a formula in which the concentrations in equation (1) are replaced by effective concentrations or activities.

Brønsted [1929], Taylor, P. B. [1927] and Guggenheim [1929] have emphasised the difficulty of measuring the potentials between two different media and the activities of individual ions, but in colloidal solutions the molar fraction of water is so nearly equal on both sides of the membrane that the conditions for the application of the formula of Donnan and Allmand are more favourable, even if absolute accuracy is unattainable on account of liquid junction potentials.

In view of the number and variety of the practical applications of measurements of membrane potentials in colloidal systems, even approximate results may be of great value, and in certain cases it is possible to compare the results with data obtained by methods that are independent of liquid junctions, including measurements of osmotic pressure and determinations of the concentrations of ions on both sides of the membrane by chemical methods.

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Determinations of p_H values, membrane potentials, combined ions and other properties of proteins deduced from measurements in which approximate elimination of liquid junction potentials has been achieved by the use of saturated solutions of potassium chloride must be regarded as conventional rather than absolute values.

The chief application of membrane potentials described in this paper is the estimation of the electric charge or valence of the protein ions from the limiting value of the ratio of the membrane potential to the protein concentration. A preliminary note on the method has been included in a previous paper [Adair and Robinson, 1930, 2].

2. *Liquid junction potentials.*

Sørensen and Linderstrøm-Lang [1924] have found that in most of the solutions that are of biological interest the elimination of the liquid junction potentials by the use of saturated potassium chloride gives the same result as the Bjerrum extrapolation. Bjerrum and Unmack [1929] have made an extensive series of calculations of the liquid junction potentials with 3·5 N potassium chloride by the application of Henderson's formula. If the concentration of ions like sodium, with mobilities different from the potassium and chloride ions, be less than 0·2 M, the potentials rarely exceed 1 millivolt, and even if hydrogen and hydroxyl ions be present in concentrations up to 0·03 M the potentials are usually within 1·2 millivolts. The choice of solvents within this range is of importance from the experimental point of view in reducing variations in the potentials due to differences in the methods of forming the liquid junctions, so that reproducible results can be obtained by methods specially adapted for measurements on small volumes of solutions. In order to obtain reproducible results with markedly acid or alkaline solutions, it may be necessary to use flowing junctions [Lamb and Larson, 1920] or static junctions in wide tubes [Ferguson *et al.*, 1932].

In previous investigations on membrane potentials, liquid junctions in capillary tips have been used by Loeb [1922] and by Taylor [1924], in order to minimise the contamination of the solution with potassium chloride. Most investigators have used wider tubes, which give more reproducible liquid junctions. Kondo [1924] formed the junction at the end of a wide tube, Bjerrum made liquid junctions in glass taps, Northrop and Kunitz [1926] formed stirred junctions in wide tubes.

Different types of liquid junctions have been discussed by Guggenheim [1930]. He criticises the practice of stirring junctions, on the ground that it forms two junctions of indefinite type between the first solution and the mixture and the second solution. He advises the use of sharp junctions formed inside tubes and not at their tips. Maclagan [1929] compared junctions formed with tubes of different diameters and concluded that junctions formed inside tubes are more reproducible.

Stadie and Hawes [1928] showed that the liquid junction formed in the Clark hydrogen electrode gives reproducible results in measurements of the p_H values of mixtures containing haemoglobin and sodium salts. This method has been employed in this work in the determinations of membrane potentials from p_H values.

3. *The experimental determination of membrane potentials.*

In the method for the measurement of membrane potentials devised by Loeb [1922], a solution of the protein contained in a 50 ml. collodion membrane, with a manometer attached, was placed in a vessel of "outer fluid" or dialysate,

and the system was left for 20 hours to reach equilibrium. The membrane potential was determined by dipping saturated calomel-KCl electrodes into the dialysate and the protein solution, which was forced up into a funnel by pressure on the flexible collodion membrane. In most of Loeb's experiments the results obtained were verified within two millivolts by their agreement with values calculated by the application of Donnan's formulae to measurements of the activities of hydrogen ions on both sides of the membrane.

In the modifications of the methods devised by Bjerrum [1924] for chromium chloride, by Kondo [1924] for caseinogen chloride and by Henriques [1928; 1929] for laked blood corpuscles, the liquids were enclosed, and long glass tubes connected the inner and outer fluids with calomel electrodes. These designs facilitate the measurement of potentials at short intervals of time; moreover, the pressures may be adjusted at the values corresponding to osmotic equilibrium. According to Rinde [1926], measurements of potentials must be made at the equilibrium pressure, whereas Northrop and Kunitz [1926] consider that it is unnecessary to maintain the equilibrium pressure during the measurement. As will be stated below, our experience agrees with that of Northrop and Kunitz.

The method for the determination of membrane potentials to be described in this paper was developed for work on purified protein solutions, in order to obtain consistent results under conditions where the potentials may be small and the volumes of solution available for each determination may be restricted to 5 or even 1·5 ml. The types of apparatus used in this work are not adapted for making continuous records of the potentials, but they have the advantage that dead spaces, which may contain protein not in equilibrium with its dialysate, are practically eliminated, and the distances for diffusion are comparatively short (Fig. 1).

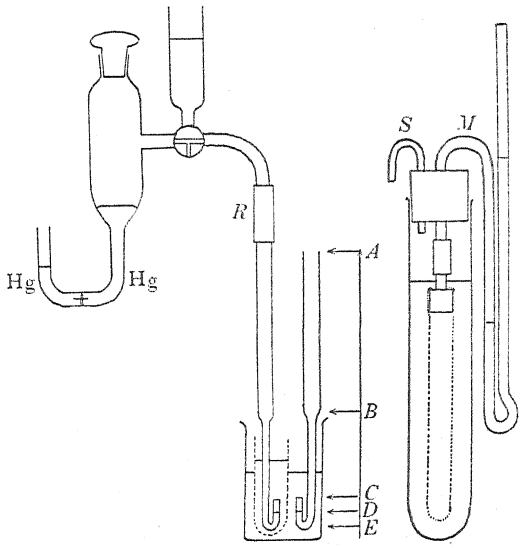

Fig. 1. Apparatus for measurement of membrane potential of small volumes of protein solutions with saturated calomel-KCl electrodes.

Collodion membrane containing protein shown by dotted lines. *R* is rubber connecting tube. Electrode tip, *A* to *B* 90 mm. tube of 5 mm. internal diameter; *B* to *E*, 55 mm. tube of 2 mm. internal diameter; *E* to *C*, 15 mm. Liquid junction at *D*, 7 mm. below orifice at *C*. *M* is mercury manometer bore 1·5 mm. attached to osmometer used for equilibration. *S* is for attachment of soda lime tube.

The establishment of equilibrium between the protein solution and the dialysate is the first consideration in studies of membrane potential. At ordinary temperatures, rapid equilibration may be necessary to prevent errors due to changes in the protein and to bacterial action. In the present research, the work throughout was carried out at 0°, and since bacterial action is inhibited and the stability of the proteins is greater at low temperatures, it has been possible to make more rigorous tests of equilibrium. Instead of leaving given volumes of protein solution and dialysate to reach equilibrium, as described by Loeb, the protein solutions were equilibrated by dialysis against relatively large volumes of solutions of well defined hydrogen ion concentration, in order to control the activities of the electrolytes in the protein solutions and to facilitate the thermodynamical treatment of the systems [Adair, 1929, 1, 2]. The standard buffer solutions remove traces of inorganic impurities which are not readily eliminated by a preliminary dialysis against distilled water; the rate of removal of traces of an acid or base combined with the protein is very greatly increased if the dialysate be a solution of electrolytes rather than pure water.

Method 1. In the majority of the experiments to be described in this paper, the process of equilibration has been followed by observation of the osmotic pressure.

The collodion membranes used were prepared from a solution containing 4 g. of dry pyroxylin (B.D.H.) dissolved in a mixture of 50 ml. of absolute alcohol and 50 ml. of anhydrous ether, or by diluting one volume of "Necol" with one volume of alcohol and one volume of ether. The membranes were made by pouring three coats of collodion over the outside of a glass mould [Adair, 1925; Cole, 1933]; they were therefore less permeable but more rigid and probably more uniform than those used by Loeb, which were made on the inside of a tube with one coat of collodion. The permeability may be altered by varying the time allowed for drying the collodion. The membranes were mounted on rubber tubes or stoppers, and the permeability was tested by measuring the rate of flow of water through the membrane at room temperature under a pressure of 450 mm. Hg. For a cylindrical membrane of 10 cm. length and 1·5 cm. diameter, the rate of flow should range from 0·05 to 0·2 ml. of water per minute. Membranes of higher permeability may allow an appreciable leakage of a protein such as haemoglobin, whereas those which are less permeable entail an unduly prolonged period of equilibration.

The collodion membranes were attached to mercury manometers of the type illustrated in Fig. 1 for concentrated solutions, and of the type described by Adair [1925] for more dilute solutions. The dialysates were changed at intervals of one or two days until further changes caused no further alterations in the manometer readings. If the volume of the dialysate be about ten times as great as the volume of the protein solution, a close approximation to equilibrium is reached after three or four changes, in the case of well buffered solutions.

If the buffer solutions used are more dilute than 0·3 M, it is advisable to dialyse the protein solutions for two or three days against smaller volumes of buffer, changed at short intervals, before the membranes are attached to manometers, and if there be a large difference between the initial hydrogen ion concentration of the protein solution and the equilibrium value, it may be advisable to adjust the p_H of the protein solution or to add a slight excess of acid or base to the dialysate in the early stages of dialysis.

The apparatus and procedure may require modification for work on very dilute solutions (0·001 M), or for solutions in which the p_H values are altered by a short exposure to air.

The determinations of membrane potential were made after equilibrium had been reached and maintained for a few days. Closed saturated calomel-KCl electrodes with upcurved tips were used, of the design shown in Fig. 1. The electrodes were kept throughout at 0° and were connected by leads to a potentiometer in the laboratory outside. A mirror galvanometer of 450 ohms resistance was used, giving a deflection of about 40 mm. per millivolt, with electrodes dipped into solutions of high conductivity. A multiple range potentiometer was used for some experiments, but as a general rule the measurements were made with a simpler instrument, graduated in 0·2 millivolt.

The difference between the two calomel cells was first determined with both electrode tips placed in a tube of saturated KCl; the electrode tips were then washed with distilled water and dried with filter-paper, and the blank determination was repeated. The two measurements should not differ by more than 0·01 millivolt, but variations of 0·03 millivolt have been observed if the calomel cells have been shaken during the manipulations. If the blank determinations agreed, the electrodes were again washed with distilled water, and a few drops of KCl were run out to displace water which might have entered the tips. A small quantity of KCl was then forced out by pressure on the rubber tube connecting the electrode tip and the calomel cell, so that the level of KCl was about 7 mm. below the orifice; the electrodes were then dried with filter-paper. After both electrodes had been prepared, an osmometer was dismantled and the protein solution filling the dead space, *i.e.* that which had not been in contact with the membrane, was rejected. The bulk of the protein was removed for analysis, a depth of solution of 2 to 3 cm. being left in the membrane. The rubber mount was then cut off, and the membrane was placed in a small beaker containing dialysate. In cases where a positive charge on the protein was anticipated, the more positive of the two calomel cells was selected, and the orifice of the electrode tip, from which KCl had been removed, was filled with the protein solution; the other electrode was filled with dialysate. Both electrodes were then washed with dialysate and dried with filter-paper. The more positive electrode was then immersed in the protein solution remaining in the membrane and the other electrode in the dialysate.

In order to avoid the formation of air bubbles in filling the electrode tips, the rubber connecting tube was pressed until KCl rose to the level of the orifice. The protein solution could then be drawn into the tip from a capillary pipette by releasing the pressure on the rubber tube.

The first reading of the potential could generally be obtained between three and four minutes after the formation of the liquid junctions. The reproducibility of the junctions formed by this method and the variation of the reading with time are discussed in the following section.

Method 2. In this method, the protein solutions were contained in collodion membranes mounted on celluloid tubes of 1·5 cm. length. During equilibration, the membranes were closed with rubber stoppers and were inverted at intervals. The attainment of equilibrium was determined by measuring the potential difference between protein solution and dialysate on successive days and renewing the dialysate each day until further changing caused no alteration in the potential. Stoppered pyrex beakers were used for equilibration.

The osmotic method is more convenient for series of more than twelve membranes, as the manometer readings can be made more rapidly than a single estimation of membrane potential. The second method is useful for the measurement of small potentials, as a number of determinations can be obtained from each membrane, and the effects of certain sources of error, including changes in

the E.M.F. of the calomel cells and variations in the liquid junction potentials, can be minimised by taking the average of a number of observations after equilibrium has been reached. Observations on the rate of equilibration of haemoglobin solutions with 0·02 *M* phosphate buffers, obtained by this method, are given in Table I.

Table I. *Rate of equilibration of 22 % solutions of sheep carbon monoxide haemoglobin with 0·01* M $NH_4H_2PO_4$ + *0·01* M $(NH_4)_2HPO_4$ *at 0°, shown by measurements of membrane potential on successive days.*

Permeabilities in ml. water per minute at a pressure of 450 mm. of mercury for membranes 4·5 cm. long and 1·4 cm. diameter. Times in days. Potentials in millivolts.

Membrane permeability	1	4	6	8	12	21	55 days
0·086	−1·35	−0·77	−0·66	−0·62	−0·54	−0·55	—
0·066	−1·20	−0·72	−0·64	−0·66	−0·46	−0·55	—
0·102	−1·15	−0·77	−0·62	−0·55	−0·51	−0·55	−0·59
0·028	−1·30	−0·77	−0·61	−0·50	−0·51	−0·50	−0·48
0·083*	+0·00	−0·35	−0·31	−0·32	−0·40	−0·29	−0·35
0·047*	+0·60	−0·30	−0·32	−0·27	−0·33	−0·27	−0·29

* Some methaemoglobin present.

4. *Preparation of protein solutions and methods of analysis.*

The proteins investigated included the haemoglobins of the sheep and the ox, horse-serum-albumin, total serum-protein, denatured edestin, acid haematin and kathaemoglobin. Some experiments were also carried out on Congo red.

The haemoglobin solutions were prepared by a slight modification of the method given by Adair [1928] which will be described in detail in a later communication. Horse-serum-albumin was recrystallised three times by the method of Adair and Robinson [1930, 1]. The total serum-protein was prepared by the dialysis of untreated serum with phosphate buffer of p_H 7·4. Denatured edestin was made by prolonged dialysis of crystalline edestin, prepared from hempseed, against 0·01 *M* HCl. Acid haematin was obtained by dialysis of haemoglobin against an acetate buffer of p_H 3·04. A soluble form of kathaemoglobin was prepared by mixing equal volumes of 0·9 *M* NaOH and a 22 % solution of haemoglobin (mainly in the form of methaemoglobin) which had been recrystallised by means of ammonium sulphate. After standing at room temperature for a few days, to ensure complete denaturation, the excess of alkali was removed by dialysis, and the alkaline haematin was converted into kathaemoglobin. In some experiments, carbon dioxide was passed through the solution before dialysis.

The Congo red was subjected to a preliminary dialysis against distilled water, in order to remove chlorides, before equilibration with sodium bicarbonate. The concentration of the solution obtained was estimated from dry weight determinations.

In all experiments on protein solutions, the concentrations were determined after equilibration by Kjeldahl nitrogen determinations or by the refractometric method, using factors based on nitrogen determinations [Adair and Robinson, 1930, 1].

The hydrogen ion concentration values of the dialysates at 0° were measured with the Clark hydrogen electrode and a saturated calomel-KCl electrode, standardised by measurements of a mixture of 0·01 *M* HCl in 0·09 *M* KCl. The conventional value $p_H = 2·04$ was assigned to this solution. In certain experiments

the p_H value of the protein solution was measured by the hydrogen electrode, but in most experiments it was calculated from the membrane potential and the p_H value of the dialysate.

The following methods were used for analysis of the dialysates: ammonia was estimated by nesslerisation or by the micro-Kjeldahl; phosphates by the method of Bell and Doisy [1920], carbon dioxide according to Van Slyke and Neill [1924], and chlorides by the method of Van Slyke [1923]. The technique for the estimation of sodium will be described in a subsequent communication.

In the analysis of the protein solutions, protein-free filtrates were obtained by heat-coagulation of diluted solutions at the isoelectric point in the presence of sodium sulphate.

In experiments with concentrated buffer solutions (0·05 *M*) complete analyses of the dialysate are not required in all cases, if the refractive indices of the stock buffer and the final dialysate be identical and no difference between their p_H values can be detected by colorimetric methods.

5. *Sources of error in measurements of membrane potentials.*

The sources of error in measurements of the membrane potentials of protein solutions may include impurities, alterations in the proteins during the process of preparation, deviations from a state of equilibrium between protein solution and dialysate due to the action of bacteria, imperfections in the membranes, changes in the dialysate and breakdown of the protein. During electrometric measurements there are additional sources of error, including alterations in the potentials of the calomel electrodes, contamination of the solutions with potassium chloride, imperfections in the method of forming the liquid junctions and, if the protein solution be not enclosed and maintained at the osmotic equilibrium pressure, there may be errors due to absorption of carbon dioxide, evaporation of water, the dilution of the protein solution and electrokinetic potentials and diffusion potentials which may be correlated with the flow of water across the membrane.

The method described in this work is restricted to solutions that are not greatly affected by carbon dioxide; during the measurements of the potentials, the solutions are exposed to the air. Experiments with buffered solutions including haemoglobin in equilibrium with 0·01 *M* phosphates, in which the solutions were left exposed to the air for several hours, indicated that the amount of carbon dioxide absorbed in the course of an hour was not sufficient to cause appreciable error. In the case of very dilute alkaline solutions, the risk of error due to carbon dioxide is much more serious, and it may be necessary to use an enclosed apparatus of the type figured by Bjerrum [1924].

In the apparatus shown in Fig. 1, the protein solutions are kept under the osmotic equilibrium pressure during dialysis, but the measurements of the potential are made with the protein solution at atmospheric pressure. Under these conditions, the dialysate flows across the membrane into the protein solution. The rate of dilution is not rapid with the type of membrane used in this work, and even if the dismantled membranes are left overnight, the final concentrations usually exceed two-thirds of the initial value. Since the first reading of the membrane potential is made a few minutes after the osmotic pressure is released, the degree of dilution is not appreciable. As a general rule, repetitions of the measurements made within the first hour agreed with the initial value, if the solutions were not contaminated with potassium chloride during the first series of readings. Agreement between the readings made after a time interval shows that the errors due to the fall in protein concentration and to

evaporation of water and absorption of carbon dioxide are not important, but it does not exclude errors due to diffusion potentials which may be associated with the movement of ions of different mobilities across the membrane and electrokinetic potentials due to the flow of fluid across a charged membrane. In neutral solutions collodion membranes are negatively charged, but the charge may be altered by adsorption of protein during equilibration.

Attempts were made to measure these effects in a system containing haemoglobin in equilibrium with 0·01 *M* phosphate buffer, using an apparatus of the enclosed type similar to that figured by Henriques [1928]. It was found that the potentials remained constant within 0·4 millivolt with arbitrary variations of pressure from 6·2 to 140 mm. of mercury.

Since the electrokinetic effects must depend upon the specific properties of the membrane, comparative experiments were set up with collodion, parchment and cellophane membranes. Discs of parchment or cellophane of about 9 cm. diameter were folded over one end of a glass tube 4 cm. long and 1·5 cm. diameter and held in position by rubber rings. The tubes prepared in this way were kept in buffer solution for one or two days; they were then filled, to a depth of about 2·2 cm., with haemoglobin solution which had been subjected to a preliminary dialysis against the same buffer solution. The tubes were placed in small beakers containing glass rods which prevented the parchment or cellophane membranes from touching the bottom of the beakers. The potentials were measured shortly after the tubes were filled with protein solution.

Table II. *Comparison of collodion, cellophane and parchment membranes.*

Exp. 618. Sheep carbon monoxide haemoglobin, 20·28 %. Osmotic pressure 93·6 mm. dialysate 0·01 *M* $NH_4H_2PO_4$ + 0·01 *M* $(NH_4)_2HPO_4$. p_H 7·046 at 0°.

Exp. A 7. Horse-serum-albumin, 15·72 %. Osmotic pressure 38·2 mm. dialysate 0·02 *M* CH_3COONa + 0·01 *M* CH_3COOH. p_H 4·99 at 0°.

Time in minutes. Potentials in millivolts.

618 Collodion		618 Parchment		618 Cellophane		A 7 Collodion		A 7 Parchment	
Time	E.M.F.	Time	E.M.F.	Time	E.M.F.	Time	E.M.F.	Time	E.M.F.
0	−1·81	0	−1·86	0	−1·78	0	−4·49	0	−4·25
2	−1·84	1	−1·91	1	−1·82	1	−4·59	2	−4·45
5·5	−1·89	3	−1·96	6	−1·91	5	−4·64	8	−4·70
9	−2·01	9	−2·04			10	−4·84	15	−4·95
15	−2·06	11	−2·06			15	−5·14	27	−5·30
21	−2·16	20	−2·10			18	−5·34	38	−5·35
31	−2·26	36	−2·06						

The results recorded in Table II show that there is a close agreement between the potentials given by different membranes, if comparisons are made at the same interval of time after the formation of the liquid junctions; no evidence is shown for the existence of errors due to specific properties of the membrane. The effects of time on the liquid junction potentials are discussed below.

Information as to the possibility of errors caused by electrokinetic potentials, diffusion potentials and imperfect equilibration may furthermore be obtained by a comparison of the observed membrane potentials with values calculated from electrometric measurements of hydrogen ion activity by a slightly modified form of the equation of Donnan and Allmand [1914].

$$E = 54{\cdot}16 \left(\frac{273+t}{273}\right) (p_{\text{H inner}} - p_{\text{H outer}}) \qquad \text{.....(2).}$$

t = temperature in ° C.

This formula has been verified by a number of investigators, including Loeb [1922] and Bjerrum [1924].

The investigations of Hastings *et al.* [1924] and of Stadie and Hawes [1928] indicated that fairly good results might be obtainable with the hydrogen electrode in solutions of carbon monoxide haemoglobin practically free from methaemoglobin. More concentrated solutions, containing 20 to 22 % of protein, used in this work, are very viscous, and the conditions are not ideal for the establishment of equilibrium with hydrogen electrodes at 0°, particularly in the case of haemoglobin. Though consistent results were obtained with most preparations, examples of which are given in Table III, in a long series of experiments the

Table III. *Direct and indirect measurements of membrane potentials of sheep carbon monoxide haemoglobin 20–22 % solutions.*

Dialysates 0·01 *M* ammonium phosphate buffers. E = membrane potential in millivolts.

Exp. no.	Dialysate p_H	Protein solution p_H	Protein solution p_H (calculated)	Direct E	Indirect E	Difference
451	6·295	6·431	6·441	+7·93	+7·3	−0·6
452	6·939	6·942	6·958	+1·03	−0·2	−1·2
454	7·096	7·054	7·089	−0·38	−2·2	−1·8
461	7·680	7·592	7·596	−4·57	−4·77	−0·2
467	6·670	6·706	6·729	+3·17	+2·06	−1·2
469	6·941	6·937	6·953	+0·62	−0·3	−0·9
475	7·398	7·321	7·341	−3·07	−4·15	−1·1
477	8·229	8·087	8·059	−9·20	−7·7	+1·5

hydrogen electrode potentials drifted. In view of the difficulties experienced with haemoglobin, a comparison of membrane potentials, measured by direct and indirect methods, was made on solutions of serum-albumin (Table IV). In

Table IV. *Direct and indirect measurements of membrane potentials of horse-serum-albumin at 0°.*

Exps. A 15, 14, 16, 9, dialysate 0·005 *M* $NH_4H_2PO_4$ + 0·005*M* $(NH_4)_2HPO_4$.
Exps. A 11, 10, dialysate 0·01*M* $NH_4H_2PO_4$ + 0·01*M* $(NH_4)_2HPO_4$.
Exps. A 7, 6, dialysate 0·02*M* CH_3COONa + 0·01*M* CH_3COOH.
Exps. A 5, 4, dialysate 0·02*M* CH_3COONa + 0·025*M* CH_3COOH.
Osmotic pressures in mm. mercury. E_1 and E_2, initial and final readings of membrane potential in millivolts. E_3 and E_4 potentials calculated from initial and final hydrogen electrode readings.

Exp. no.	Protein g. per 100 ml.	Osmotic pressure	Dialysate p_H	Protein solution p_H	Direct E_1	Direct E_2	Indirect E_3	Indirect E_4
A 15	1·95	7·1	7·056	7·005	− 2·75	− 2·75	− 2·75	− 2·56
A 14	3·36	13·8	7·055	6·963	− 4·47	− 4·54	− 5·05	− 5·05
A 16	5·38	27·3	7·054	6·911	− 6·92	− 7·12	− 7·80	− 7·55
A 9	15·92	171·2	7·041	6·640	−19·0	−20·3	−21·6	−20·2
A 11	15·15	133·6	6·987	6·742	−11·78	−12·58	−12·26	−11·16
A 10	19·09	211·7	7·001	6·709	−14·59	−15·59	−15·8	−15·8
A 7	15·45	38·2	4·985	4·900	− 4·5	− 5·6	− 4·9	− 4·8
A 6	20·06	56·4	4·979	4·867	− 5·38	− 6·68	− 6·1	− 5·9
A 5	15·87	64·6	4·588	4·607	+ 1·2	+ 0·2	+ 1·1	+ 1·0
A 4	19·99	91·2	4·582	4·603	+ 1·47	+ 0·33	+ 1·1	+ 1·7

dilute solutions of serum-albumin, there is a satisfactory agreement between the potentials determined by both methods. In solutions containing more than 15 % of the protein the experimental difficulties were greater on account of the effects at the liquid junctions, discussed below. The average discrepancy between

the initial readings of observed and calculated potentials was 0·6 millivolt, which corresponds to 0·01 p_H unit. The maximum discrepancy was 1·4 millivolts.

Traces of carbon dioxide, absorbed during dialysis, may cause small errors in indirect determinations of membrane potentials by means of hydrogen electrode measurements, because varying amounts of carbon dioxide may be removed by bubbling hydrogen through the liquids. The p_H changes due to loss of carbon dioxide are not identical in the protein solution and in the dialysate on account of the buffer action of the proteins. The agreement between the direct and indirect determinations, shown in Tables III and IV, is evidence against the existence of serious errors due to carbon dioxide.

The magnitude of the experimental errors due to liquid junction potentials depends upon the composition of the solutions under examination. The reproducibility of the liquid junctions formed in the electrodes illustrated in Fig. 1 was tested by measuring the potential difference between two electrodes dipping into the same solution. In the case of dilute buffer solutions the potentials agreed, but in concentrated (M) ammonium phosphate buffers there were variations of 0·5 millivolt. If the initial measurements were reproducible, the constancy of the potentials could be investigated by renewing the liquid junction in one of the electrodes at known intervals of time. In 0·01 M ammonium phosphate buffers, where it is possible to obtain initial values agreeing within 0·03 millivolt, the potential of the electrode left undisturbed in the solution remained constant within 0·03 millivolt for 30 minutes. In dilute solutions of proteins equilibrated with 0·01 to 0·05 M buffer solutions, the initial readings were reproducible but the variations with time might exceed 0·1 millivolt.

In solutions containing 20 % of haemoglobin, the results were reproducible if both junctions were formed within 30 seconds, but a freshly-formed junction was more positive than one left standing for a few minutes. The differences ranged from 0·2 to 0·5 millivolt, in cases where the first junction had been left standing for 15 minutes. In concentrated solutions of serum-albumin, the initial readings agreed, but the difference between a freshly formed and an older junction ranged from 1·0 to 1·3 millivolts.

Comparisons made between electrodes with wide and narrow tips showed little difference in the case of buffers and of haemoglobin solutions, but there were differences ranging from 0·2 to 0·6 millivolt in the case of serum-albumin.

It is possible that these variations with time are correlated with an alteration at the boundary between solution and saturated potassium chloride, which has been observed not only with this type of electrode but with liquid junctions formed by means of taps. At first the boundary is sharp, but within a few minutes it becomes irregular. The disturbance is probably due to convection currents, because the mixture formed by the diffusion of potassium chloride into the protein solution may be denser than the pure potassium chloride solution below it.

In a measurement of the membrane potential, the observed potential E can be regarded as the sum of E (KCl.Pr), the liquid junction potential at the boundary between saturated potassium chloride and protein solution, E_m, the potential at the membrane and E (D.KCl), the potential at the junction of the dialysate and the potassium chloride solution of the second electrode. The observations recorded in Table II show that the final values of E are more negative than the initial values, and the direction and magnitude of the changes are in accordance with those observed in studies of the liquid junction E (KCl.Pr), a result which might be expected if the potentials E_m and E (D.KCl) remained constant.

Observations of the potentials at different times after the formation of the liquid junctions are furthermore of value in detecting errors due to contamination of the solutions with potassium chloride, which may cause drifts in the potentials which are larger and more rapid than those due to liquid junction potentials. Rapid drifts may also be caused by local differences in the concentration of ions due to imperfect equilibration. It is advisable to reject experiments which show drifts in the potential which cannot be accounted for by liquid junction potentials. With dilute solutions of proteins, the potentials should remain constant within 0·1 millivolt.

In many problems, the accuracy of the ratio E/C, where C is the protein concentration, is more important than the accuracy of the potential E, and therefore an error of 0·5 millivolt on a 25 % solution is comparable with an error of 0·02 millivolt on a 1 % solution.

With the exception of the experiments in Tables II and IV, which exemplify the maximum variations with time, the potential values recorded graphically and in the Tables are those obtained between 3 and 4 minutes after the formation of the liquid junction in the protein solution. The practice of recording initial readings in measurements on inorganic solutions was adopted by Lewis *et al.* [1917] and by Clark [1928]. According to Maclagan [1929] and Guggenheim [1930], the final readings, obtained 10 or 15 minutes after the formation of the liquid junction, are more reproducible in the case of junctions formed with hydrochloric acid inside tubes.

In certain of our experiments, the final as well as the initial readings were reproducible, but it is more difficult to obtain satisfactory final readings on account of contamination of the solutions with potassium chloride. It is possible that in the case of protein solutions the initial readings represent a closer approximation to a sharp junction, and it is by no means certain that the final readings are of greater significance.

As the initial readings of membrane potentials that we have obtained for solutions of different protein concentration are reproducible, correction factors could be determined if a standard type of liquid junction for protein solutions were to be devised at a future date.

Henriques [1929], who used an electrometer, drew attention to polarisation as a possible source of error in measurements of membrane potentials by the use of a galvanometer and a potentiometer. In this work it was found that there was no difference between the potentials measured just before and immediately after attempts to polarise the system by closing the circuit with the potentiometer set at 5 or 10 millivolts above or below the equilibrium value, causing galvanometer deflections from 57 to 112 mm.

6. *Measurements of membrane potentials at different protein concentrations in solutions where the composition of the dialysate is kept constant.*

The observations recorded in Figs. 2 to 6 show that in all the protein systems investigated, which included proteins such as haemoglobin and serum-albumin with particles of uniform size and denatured proteins which may be mixtures of particles of different sizes, there is a straight line relationship between the membrane potential and the "corrected percentage concentration," C_v, defined by formula 3, under conditions in which the membrane potential is small.

The results of analyses of the protein solutions by volumetric or by refractometric methods are usually expressed in terms of C, the concentration of dry

protein in g. per 100 ml. of solution, and the values of C_v are calculated from these measurements.

$$C_v = C\,[100/(100 - V_1 C)] \qquad \ldots\ldots(3).$$

C_v = g. of dry protein per 100 ml. of solvent. C = g. of dry protein per 100 ml. of solution. V_1 = volume occupied by 1 g. of dry protein.

The volume of V_1 cannot be less than 0·75 ml., the specific volume of the dry protein, and it may be more if the protein combines with water.

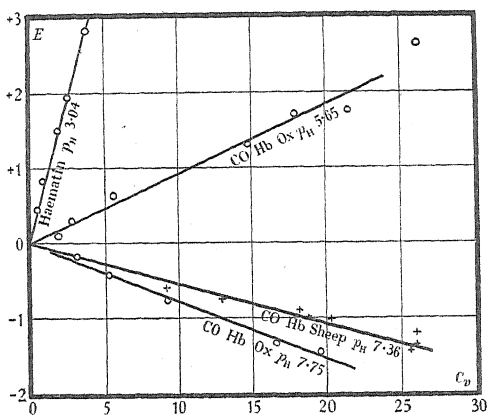

Fig. 2. Membrane potentials of CO-haemoglobin and acid haematin at different concentrations.

Dialysates of ionic strength 0·01, compositions given in mols. per litre:

p_H 3·04. CH_3COONa 0·0025, CH_3COOH 0·0975, NaCl 0·0975.
p_H 5·65. CH_3COONa 0·100, CH_3COOH 0·0105.
p_H 7·36. $(NH_4)_2HPO_4$ 0·03, $NH_4H_2PO_4$ 0·01.
p_H 7·75. Na_2HPO_4 0·0321, KH_2PO_4 0·00357.

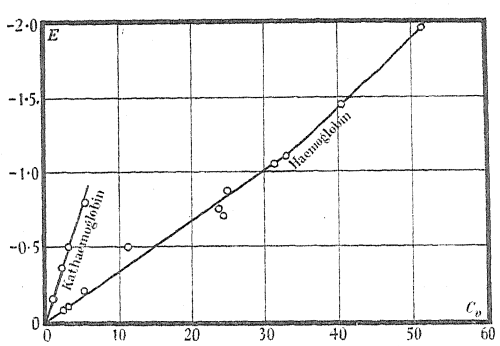

Fig. 3. Membrane potentials of haemoglobin (mixture of oxygenated and reduced) and kathaemoglobin from sheep blood.

Dialysate p_H 7·8, ionic strength 0·289, KCl 0·1, Na_2HPO_4 0·0613, KH_2PO_4 0·00533.

The hydration of haemoglobin, estimated from chloride distribution measurements, is 0·21 g. per g. of dry protein [Adair, 1928]. The hydration of the haemoglobin of the horse in the presence of ammonium sulphate has been investigated by Sørensen and Sørensen [1933]. The value 0·353 g. given in their paper is reduced to 0·306 g. if the calculation is based on the nitrogen determination of 16·69 %, made by Vickery and Leavenworth [1928], rather than the

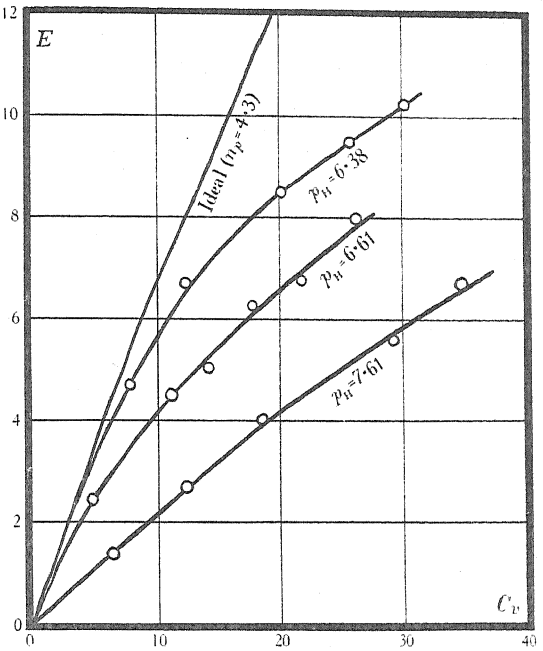

Fig. 4. Membrane potentials of sheep CO-haemoglobin.

"Ideal" $n_p = 4{\cdot}3$ curve calculated for a protein of valence 4·3 in ideal solutions. Dialysates of ionic strength approximately 0·01.

p_H 6·38. $(NH_4)_2HPO_4$ 0·00128, $NH_4H_2PO_4$ 0·00672.
p_H 6·61. $(NH_4)_2HPO_4$ 0·0017. $NH_4H_2PO_4$ 0·0053.
p_H 7·61. $(NH_4)_2HPO_4$ 0·0037. $NH_4H_2PO_4$ 0·0012.

At p_H 7·61 potentials are negative.

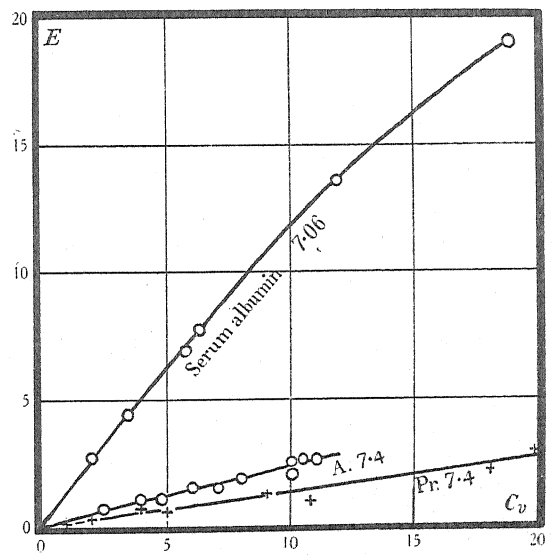

Fig. 5. Membrane potentials of serum-proteins.

Serum-albumin, p_H 7·06. Dialysate $(NH_4)_2HPO_4$ 0·005, $NH_4H_2PO_4$ 0·005. A 7·4 serum-albumin, Pr 7·4 total protein. Dialysate p_H 7·4. Na_2HPO_4 0·0533, KH_2PO_4 0·0133.

older value of 17·3 %. Adair and Robinson [1931] used a direct method, in which the dried haemoglobin of the sheep, exposed to the vapour of saturated ammonium sulphate solution, took up 0·2 g. of water per g. dried protein. Weber and Nachmannsohn [1929] estimated that the effective volume $V_1 = 1{\cdot}0$ ml. in the case of serum-albumin. In the calculations given below, the volume $V_1 = 1{\cdot}0$ has been used for serum-albumin and $V_1 = 0{\cdot}965$ for haemoglobin and its derivatives. Although these values may require correction when more complete

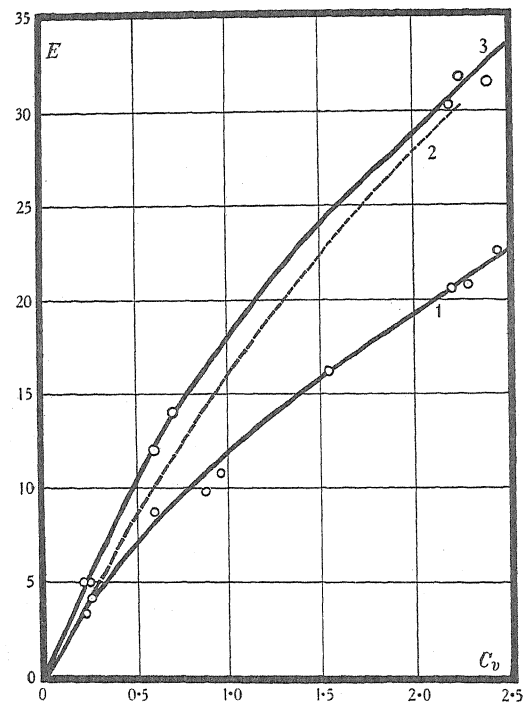

Fig. 6. Membrane potentials of edestin. Dialysate 0·0104 *M* HCl. Curve 1, observed potentials. Curve 2, theoretical potentials for protein of constant valence 26·3. Curve 3, potentials calculated from chloride concentrations (Section 8).

data are available, the straight line relationships between the membrane potentials and C_v shown in Fig. 2 indicate that the corrections are small, although it is not impossible that a straight line might be obtained in a system where the error due to an underestimate of the hydration value is compensated by a reduction in the membrane potentials in concentrated solutions, due to the interionic forces described by Debye and Hückel [1923].

7. *Relationships between membrane potentials and protein concentrations in ideal solutions.*

Bjerrum [1924] showed that in an infinitely dilute solution of a colloid equilibrated with hydrochloric acid, the potential is proportional to the concentration of the colloid in mols. per litre of solution.

The calculations given below show that in ideal solutions the potentials are proportional to the concentrations in g. mols. per litre of solvent, under certain

conditions which can be defined by the application of the formula (4), due to Donnan and Guggenheim [1932], together with the additional calculations recorded in Table V.

$$(P'-P'')\,v_i\left(1-\kappa_i\frac{P'+P''}{2}\right)+RT\,ln\frac{N_i'}{N_i''}=\frac{-n_iFE}{1000} \qquad \text{.....(4).}$$

R, T, F and E are defined as for formula (1). v_i = the volume of the ion in litres per g. mol. κ_i = the compressibility of the ion. n_i = the valence (negative for anions). N_i' = the molar fraction of the ion in the protein solution. P' = the hydrostatic pressure of the protein solution. N_i'' = the molar fraction of the ion in the dialysate. P'' = the hydrostatic pressure of the dialysate.

In all the formulae given below, where it is necessary to distinguish between the protein solution and the dialysate, the same notation is used. The symbols with two accents refer to the dialysate.

The effects of hydrostatic pressure, discussed by Hückel [1925] and represented by the first term in formula (4), are very small in colloidal solutions with low osmotic pressures. According to Kameyama [1925], a pressure of one atmosphere is equivalent to a correction of 0·06 % in the distribution of potassium chloride across a membrane, a correction which can be neglected in comparison with the experimental errors in the measurements of the molar fraction of the ions.

In aqueous solutions, the absolute values of the molar fractions are uncertain, on account of the polymerisation of water, but it is possible to calculate conventional values by formula (5), assuming that 1000 g. of water equal 55·51 mols. [Lewis and Randall, 1923].

$$N_i'=x_i'/(55{\cdot}51+S') \qquad \text{.....(5).}$$

x_i' = the molality of the ion in g. mols. per 1000 g. of water in the protein solution. S' = the sum of the molalities of all solutes including the protein present in the solution.

A similar formula $N_i''=x_i''/(55{\cdot}51+S'')$ can be applied to the dialysates. The sum of the molalities S'' is less than S', because the dialysate does not contain protein.

If, however, the difference between S' and S'' is of the order of 0·01 g. mol. per 1000 g. of water, the denominators in the formulae differ by less than 0·03 % and it follows that formula (4) is equivalent to the simpler formula:

$$x_i'\ (\text{ideal})=x_i''r_i \qquad \text{.....(6).}$$

r_i = the ideal distribution ratio, defined by formula (7).

$$r_i=\text{antilog}\,(-2{\cdot}303\,n_iFE/1000RT) \qquad \text{.....(7).}$$

At 0°, $2{\cdot}303\,n_iFE/1000\,RT=n_iE/54{\cdot}16$.

The conditions under which formula (6) is valid can be determined from measurements of the osmotic pressures of the solutions with collodion membranes impermeable by proteins. Sørensen [1917] pointed out that the osmotic pressure of an ideal solution of a protein, in which the molar fraction of the protein is small, is determined by formula (8).

$$p\ (\text{ideal})=RTm_p \qquad \text{.....(8).}$$

m_p is the corrected concentration of the protein, expressed in g. mols. per litre of dispersion medium. If p (ideal) be expressed in mm. mercury at 0°, then RT, expressed in mm. mercury and litres, is equal to $760\times22{\cdot}4=17{,}033$ at 0°. In dilute aqueous solutions at 0°, there is little difference between the corrected concentration and the molality, and if the osmotic pressure be below 170 mm. mercury, the difference between S' and S'' cannot exceed 0·01.

It is convenient to use the same units in calculations of osmotic pressures and of the distribution of ions, namely, the corrected concentrations, which are correlated with concentrations and molalities by formula (9).

$$m_i' = c_i' \ (100/100 - V_1 C) = x_i'/w' \qquad \text{.....(9).}$$

m_i' = the corrected concentration of the ion in g. mols. per litre of solvent in the protein solution. c_i' = g. mols. per litre of protein solution. V_1 and C = effective volume and concentration of protein (formula (3)). w' = kg. of water per litre of solvent.

In the dialysate, the volume of solvent, defined above, is equal to the volume of the solution, and therefore $m_i'' = c_i'' = x_i''/w''$, where w'' is the water content of the dialysate. The terms w'' and w' are almost identical in a system where the molar fraction of water in the protein solution is 99·98 % of its value in the dialysate, a value corresponding to an osmotic pressure of about 170 mm. mercury, and therefore formula (10) can be applied to solutions with low osmotic pressures.

$$m_i' \text{ (ideal)} = m_i'' r_i = c_i'' r_i \qquad \text{.....(10).}$$

Further steps in the calculation of the relationship between the membrane potential and the protein concentration are shown in Table V.

Table V. *Relationship in ideal solutions between membrane potentials and the equivalent concentration of the protein, equilibrated with salts of different valence types.*

Type (1–1). Example Pr.Cl + HCl. Type (2–1). Example $Pr.HPO_4 + Na_2HPO_4$. Type (1–2). Example $NaPr + Na_2HPO_4$, potentials negative. Type (1–1·5). Example $NaPr + Na_2HPO_4 + NaH_2PO_4$. $\Delta p_H = p_H$ difference between protein solution and dialysate.

E	Δp_H	$\frac{[Cl]_c'}{[Cl]''}$	$\frac{[H]_c'}{[H]''}$	u	Type (1–1) $\frac{m_p n_p}{J}$	Type (2–1) $\frac{m_p n_p}{J}$	Type (1–2) $\frac{m_p n_p}{J}$	Type (1–1·5) $\frac{m_p n_p}{J}$
1·18	0·022	1·051	0·951	0·05	0·050	0·051	0·049	0·049
2·35	0·043	1·105	0·905	0·10	0·100	0·105	0·095	0·096
4·72	0·087	1·221	0·819	0·20	0·201	0·224	0·184	0·188
7·05	0·130	1·350	0·741	0·30	0·305	0·360	0·267	0·276
9·40	0·174	1·492	0·670	0·40	0·411	0·518	0·348	0·363
11·75	0·215	1·649	0·606	0·50	0·521	0·704	0·427	0·450
16·45	0·304	2·014	0·497	0·70	0·759	1·695	0·589	0·631
23·54	0·434	2·718	0·368	1·00	1·175	2·340	0·861	0·940

In making these calculations, it is assumed that the experimental data given are the membrane potentials and the concentrations of the ions in the dialysate, but as all the terms given in the tables are functions of the membrane potentials, there are many alternative sets of data that could be utilised. The first column gives the membrane potentials in millivolts at 0°, the temperature at which the measurements were made in this work. For comparison with Loeb's measurements at 24°, the potentials should be multiplied by (273 + 24)/273. The terms in the other columns require no temperature corrections. The p_H difference between protein and dialysate is recorded in the second column. The third column gives the ideal distribution ratio for chloride ions, which may be calculated by formula (10) or by the exponential formula stated below:

$$r_i = e^{-n_i u} = [1 - n_i u + \tfrac{1}{2} n_i^2 u^2 - \tfrac{1}{6} n_i^3 u^3 + \ldots] \qquad \text{.....(11).}$$

$u = E \times F/1000RT = E/23{\cdot}535$ at 0°.

The fourth column gives the ideal distribution ratio for the hydrogen ions. Since the concentrations of the ions in the dialysate, symbolised [H]″ and [Cl]″, are known, their corrected concentrations in the protein solution, symbolised $[H]_c'$ and $[Cl]_c'$, can be calculated from the distribution ratios. The equivalent concentration of the protein chloride is then calculated as the difference between $[Cl]_c'$ and $[H]_c'$, and if m_p, the corrected concentration of the protein ions, is known, the valence, or more precisely the mean valence of the protein ions, defined as the ratio of the equivalent and the molar concentrations, can then be calculated by formula (12).

$$m_p n_p = [Cl]_c' - [H]_c' \qquad \text{.....(12).}$$

If the exponential series in formula (11) is applied, the term $[Cl]_c'$ is equal to $[Cl]''(1+u+\frac{1}{2}u^2)$, and $[H]_c'$ equals $[H]''(1-u+\frac{1}{2}u^2)$ and if u be small, so that higher powers of u can be neglected, $m_p n_p$ is equal to $u([Cl]''+[H]'')$.

Formula (12) differs from the equations given by Bjerrum [1924] and by Adair [1928] in that c_p is replaced by m_p. Similar calculations show that if the dialysate contains a mixture of ions of different valences, $m_p n_p$ is determined by formula (13).

$$m_p n_p = -\Sigma m_i' n_i \qquad \text{.....(13).}$$

$\Sigma m_i' n_i$ = the sum of the corrected concentrations of the ions in the protein solution, multiplied by their valences. The ideal values of m_i' are determined by formula (10). If u is small, formula (14) can be applied:

$$m_p n_p = uJ \qquad \text{.....(14).}$$

J = the sum of the concentrations of the ions in g. mols. per litre of dialysate, multiplied by the squares of their valences. Both n_p and u are negative when the protein is acting as an anion.

In Table V, exact values of the ratio $m_p n_p/J$ have been calculated, not only for univalent ions but also for mixtures such as the phosphate buffers containing bivalent ions, by the application of formulae (13) and (10), using tables of values of e^u and e^{-u} to calculate the distribution ratios referred to in formula (11). The simpler formula (14) can be applied over the range where the difference between u and $m_p n_p/J$ is small.

8. *The determination of the valence of protein ions.*

Pauli and Valkó [1933] have discussed the difficulties inherent in estimations of the valence of protein ions and have given references to previous investigations bearing on this problem. The method of cataphoresis is of historical interest, because the existence of a charge on protein ions, which depends upon the hydrogen ion concentration, was first demonstrated by measurements of the mobilities of the proteins in an electric field [Hardy, 1899; 1905].

The calculation of the charge presents considerable difficulties, on account of the uncertain effects of the "atmosphere" of inorganic ions surrounding the colloidal particle, referred to by Tiselius [1930], who has made most accurate measurements of the mobilities of proteins, and by Abramson [1933].

All other methods depend upon estimates of the concentrations of the inorganic ions present in the free state in the protein solution and the application of a formula of the type stated below:

$c_p n_p$ = sum of equivalents anions − sum of equivalents cations .....(15).

The concentrations of the ions in the free state cannot be determined directly; the results obtained by chemical analyses represent the sum of free and combined ions; the activities of the ions measured by electrometric methods may be different from the true concentrations, unless the solutions are so dilute that it is possible to neglect the interionic forces described by Debye and Hückel [1923]. Similar difficulties arise in estimates based upon osmotic pressures, conductivities and membrane potentials of the solutions.

The method described in this work is based on measurements of membrane potential, but it differs in certain respects from the methods described by Bjerrum [1924], Rinde [1926] and other investigators. Firstly, the measurements are made on solutions with relatively small membrane potentials and secondly, the relationship between the potential and the protein concentration is determined under conditions where the composition of the dialysate is kept constant, as shown in Figs. 2 to 6.

In the theoretical interpretation of the curves, it must be remembered that the pure salt solutions of the dialysates do not follow the ideal solution laws. The formula of Donnan and Allmand, discussed in Section 1, for membrane potentials in non-ideal solutions, can be stated in the form $a_i' = a_i'' r_i$, where a_i' and a_i'' denote the activities of the ion in the protein solution and in the dialysate, or in the alternative form $x_i' f_i' = x_i'' f_i'' r_i$, where f_i' and f_i'' denote the activity coefficients of the ions; it follows that $m_i' (f_i'/f_i'') = m_i'' r_i$ (formula (16)) within the range of application of formulae (6) and (10).

According to the theory of Debye and Hückel [1923], the activity coefficient is a function of the ionic strength of the solution, which is equal to $\frac{1}{2}J$. The calculations recorded in Table V show that in systems with large membrane potentials, there is a marked excess of ions inside the membrane. The difference between the values of J' and J'' in the protein solution and in the dialysate must affect the activity coefficients. If the membrane potential be less than 2 millivolts, the excess of ions inside the membrane is relatively small, and unless there are specific effects due to the protein, the ration f_i'/f_i'' should approach unity. If this ratio be equal to unity at very low protein concentrations, formula (16) is the same as formula (10), and it follows that the straight line relationship shown by formula (14) and by Table V can be applied to the non-ideal as well as the ideal solution. The valence of the protein ions is then determined by formula (17).

$$n_p = \left(\frac{u.J}{m_p}\right)_0 = 0{\cdot}00425\, M.J \left(\frac{E}{C_v}\right)_0 \qquad \text{.....(17).}$$

0·00425 is calculated for a temperature of 0° using the constants in formula (11). M = the molecular weight of the protein. $(E/C_v)_0$ = the value of the ratio (E/C_v) at $C_v = 0$, estimated by extrapolation from a series of measurements in which the composition of the dialysate is kept constant. $C_v = m_p . M/10$.

An experimental test of the formula (17) has been made by determining the relationships between E and C_v for a number of different proteins and a number of different solvents, covering a p_H range from 2·0 to 7·8 and ionic strengths from 0·01 to 0·287. In all the systems investigated, there is a straight line relationship between E and C_v over a range from 0 to 2 millivolts, as shown in Figs. 2 to 6, a result in accordance with the assumptions made in the derivation of formula (17).

Deviations from the ideal relationships are observed only in solutions where the membrane potentials are large as in the case of haemoglobin phosphate (Fig. 4) and edestin chloride (Fig. 6), but under these conditions there is a marked excess of ions inside the membrane which must alter the activity coefficients.

A part of the difference between the ideal and the observed curves for haemoglobin phosphate can be accounted for by the difference between the p_H values of the protein solution and the dialysate and the effects of the p_H on the valence, a subject which will be discussed in a subsequent communication.

Although the results recorded in Figs. 2 to 6 are all consistent with formula (17) and show that n_p is constant in solutions with low membrane potentials, they do not prove that the absolute value of n_p is the same as that calculated by formula (17). Evidence bearing on the absolute value of n_p is given below, but the results cannot be regarded as decisive because all alternative methods are subject to uncertainty.

Congo red and 0·02 M *$NaHCO_3$*. The determination of the valence of Congo red from measurements of membrane potential is of interest because in the case of this colloid, independent evidence concerning the degree of ionisation has been obtained by osmotic methods. Bayliss [1911] and Donnan and Harris [1911] obtained osmotic pressures about one-third of those calculated for a fully ionised disodium salt Na_2R, and Donnan and Harris showed that the observed pressures could be accounted for if Congo red ionised according to the equation

$$(Na_2R)_n = n\,Na + (NaR)_n,$$

where n is the number of molecules present in the colloidal micelle of Congo red. The electrical conductivities indicate greater ionisation, but this difference may be accounted for by McBain's theory that the ionic micelles have a relatively high mobility [McBain *et al.*, 1919].

The data recorded in Table VI show that the equivalent concentration $m_p n_p$, deduced from the membrane potentials, is from 47 to 53 % of the total sodium equivalent to Congo red, a result in accordance with the osmotic measurements of Donnan and Harris.

Table VI. *Membrane equilibrium of Congo red at 0°. Dialysate 0·02* M *$Na_2CO_3 + NaHCO_3$*. p_H *9.*

All concentrations expressed in millimols. per litre, except that of Congo red, given in milli-equivalents.

$[CO_2]''$ = sum of $[HCO_3]'' + [CO_3]''$. Proportions of ions determined by formula $[Na]'' = (1 + x'')[CO_2]$, where x'' = fraction of total CO_2 present as carbonate in the dialysate. $[CO_2]'$ = total CO_2 in the colloidal solution. $[CO_2]_c'$ (calc.) computed from values of $[CO_2]''$, x'' and E by application of formula $m_i' = m_i'' r_i$. $-m_p n_p$ = corrected equivalent concentration of Congo red computed from formula $-m_p n_p = [Na]_i'$ (ideal) $- (1 + x')[CO_2]$ (ideal). x' = fraction of total CO_2 present as carbonate. Values for [Congo red] represent total sodium equivalent to Congo red, determined by analyses of $[Na]'$ and $[CO_2]'$ and the calculation of x'. Observed values of [Congo red] $[Na]'$ and $[CO_2]'$ uncorrected because of the low concentrations of Congo red (0·51 to 1·79 g. per 100 ml.).

Dialysate		Inner fluid					
$[Na]''$	$[CO_2]''$	[Congo red]	$[Na]'$	$[CO_2]'$	$[CO_2]_c'$ calc.	$-m_p n_p$	E
20·2	18·9	14·4	30·8	15·5	15·6	7·7	− 4·21
20·1	18·9	22·0	37·1	14·4	14·5	10·5	− 5·87
19·9	19·0	24·0	38·4	13·9	14·3	11·3	− 6·42
19·9	19·0	25·9	40·1	13·8	13·5	13·1	− 6·92
20·2	18·9	32·8	45·8	12·4	12·6	16·4	− 9·04
20·2	19·2	50·3	61·3	10·7	10·8	23·7	− 12·92
20·2	19·2	51·0	61·7	10·4	10·6	24·8	− 13·48

Edestin chloride and 0·0104 M *HCl*. The ratio $(E/C_v)_0$, calculated from the experiments on edestin recorded in Fig. 6, is equal to 17·5, and making the provisional assumption that in a solution of edestin denatured by hydrochloric

acid, the mean molecular weight is 17,000, the mean valence is 26·3. An approximate confirmation of this value is afforded by the determinations of the chloride concentrations on both sides of the membrane by chemical analyses. The curve marked 3 in Fig. 6 gives the potentials calculated from these analyses by the application of formulae (7) and (10) on the assumption that the protein chloride is fully ionised. Even in the most concentrated solutions, the observed potentials are about 70 % of the calculated values.

A rough estimate of the valence of edestin as 24·0 has been made on the assumptions that the chloride is fully ionised and that the activity coefficient of the hydrogen ions is the same as that of the chloride ions [Adair, 1928]. The electrometric measurements of Hitchcock [1923] on an edestin preparation not exposed to prolonged dialysis with acid give the slightly smaller value of 21. The criticism published by Stadie and Sunderman [1931] of the value 24·0 is based on a misunderstanding. They overlook the fact that in solutions of protein chlorides equilibrated with free hydrochloric acid, it is necessary to deduct the free hydrochloric acid from the total chloride in order to estimate the chloride equivalent to the protein. In their criticism of Adair's conclusion that an increase in the concentration of edestin diminishes the activity coefficient of the chloride ions, they point out that the electrometric measurements involved liquid junction potentials. It is unlikely that the change in the activity coefficient can be accounted for by the liquid junction potentials, because the protein diminishes not only the activity coefficients but also the osmotic coefficient g' defined by the formula $\pi = RTg'$ ([H]+[Cl]+[Pr]), where π is the total osmotic pressure. In solutions containing 0·246, 0·60 and 2·16 g. of protein per 100 ml. the values of g' were 0·94, 0·85 and 0·73 respectively.

Serum-albumin. The potentials of serum-albumin recorded in Table VIII are relatively large, and instead of determining the ratio $(E/C_v)_0$ by extrapolation, the "apparent valence" at each protein concentration was calculated, as shown in Table VIII. The limiting value for $C_v=0$, determined by extrapolation, is $-17{\cdot}1$. The apparent valences diminish as the protein concentration increases, but in all cases they exceed the values of y, the number of ammonium ions equivalent to the protein determined by chemical methods. The values of y are subject to error on account of the technical difficulties of the analyses, but the difference between n_p and y exceeds the experimental errors.

In a pure aqueous solution of an ammonium proteinate, the value of n_p cannot be greater than y, and it may be less if the proteinate be not fully ionised. In the presence of neutral salts, it is theoretically possible for n_p to exceed y, if the protein exists as a zwitterion, with many positive and negative charges, and if the anions of the neutral salt are capable of combining with, or neutralising some of the positive charges of the zwitterion. In the case of a zwitterion, n_p represents the difference between the numbers of positive and negative charges, and it is possible to increase n_p without altering the amount of acid or base combined, as determined by titration curves and by analyses of the type recorded in Table VII. This hypothesis is open to objection on chemical grounds, because the salts of ammonia and its simpler derivatives appear to belong to the class of strong electrolytes in aqueous solutions, and therefore a compound of the type $R\,NH_3Cl$ should be fully ionised [Sidgwick, 1927]. The evidence is not decisive, because a protein may have different types of positive groups.

If unionised phosphate be present associated with the protein, the observed concentration of the phosphates (m_i') should exceed the ideal value.

$$m_i' \text{ (observed)} = m_i'' r_i + b m_p \qquad \text{.....(18).}$$

The term b represents the apparent combination of ions with the protein, the sum of b_1, the number of ions combined with one molecule of the protein and of b_2, which represents the excess of ions in the neighbourhood of the protein which may be attracted by the interionic forces. Formula (18) differs from an equation published by Northrop and Kunitz [1926] in that a correction for the hydration of the protein is made in the calculation of m_i' by formula (18), and that b is regarded as the sum of two terms and is expressed in corrected concentrations rather than molalities.

The observations recorded in Table VII showed differences between the ideal and the observed concentrations of ammonium ions scarcely greater than the experimental errors. Theoretically, the ammonium proteinate tends to diminish the activity coefficients, but the relatively low concentration of the bivalent ion HPO_4 in the protein solution acts in the opposite direction. The observed concentrations of total phosphate are greater than the ideal concentrations, and the values of b recorded in Table VIII show an excess of about four phosphate molecules per molecule of albumin in dilute solutions, which is sufficient to account for the difference between y and n_p, on the assumption that the phosphate is unionised. The estimate of b, the apparent combination of phosphate ions with the protein, is affected by errors in the electrometric measurements as emphasised by Stadie and Sunderman [1931], but the chemical measurements, given in Table VII, show that the product of the corrected con-

Table VII. *Membrane equilibrium at 0° of serum-albumin and 0·005* M $NH_4H_2PO_4 + 0{\cdot}005$ M $(NH_4)_2HPO_4$.

Mean p_H of dialysates after equilibration = 7·06. All concentrations in millimols. per litre solvent. m_p = corrected concentration of albumin, assuming mol. wt. 72,000. $[PO_4]''$ = total phosphate in dialysate = $HPO_4 + H_2PO_4$. x' = fraction of total phosphate in protein solution in bivalent form, computed from $[NH_4]''$, $[PO_4]''$ and E.

m_p	$[NH_4]''$	$[NH_4]_c'$ ideal	$[NH_4]_c'$ observed	$[PO_4]''$	$[PO_4]_c'$ ideal	$[PO_4]_c'$ observed	x'
0·287	15·11	17·00	17·50	10·07	8·47	9·71	0·471
0·482	14·95	18·08	18·50	9·73	7·31	9·61	0·489
0·792	14·80	19·85	20·6	10·15	6·70	9·73	0·386
0·882	15·15	21·11	20·3	10·17	6·30	9·15	0·408
1·645	14·75	26·20	27·9	10·00	4·46	8·86	0·339
3·320	15·00	41·20	37·4	10·00	2·48	9·15	0·267
2·480*	29·90	49·20	48·6	20·40	10·12	19·78	0·346
3·280*	29·97	55·70	48·9	20·00	8·28	19·80	0·350

* 0·02 *M* phosphate buffer.

Table VIII. *Observed membrane potentials of serum-albumin and ammonium phosphate at 0°.*

n_p, the apparent valence, computed from data in Table VI by formula $-m_p n_p = [NH_4]_c'$ (ideal) $-(1+x')[PO_4]_c'$ (ideal). y = base per mol. protein, computed from formula $m_p y = [NH_4]_c'$ (observed) $-(1+x')[PO_4]_c'$ (observed). b = excess PO_4 per mol. protein, defined by formula (18). p = osmotic pressure in mm. mercury. p_H represents the protein solution.

m_p	E	p	p_H	n_p apparent	y	b
0·287	− 2·75	7·1	7·00	− 15·8	11·2	4·3
0·482	− 4·47	13·8	6·96	− 14·9	8·7	4·8
0·792	− 6·92	27·3	6·91	− 13·4	9·0	3·8
0·882	− 7·78	32·5	6·88	− 13·9	8·4	3·2
1·645	− 13·56	90·7	6·80	− 12·3	9·9	2·6
3·320	− 23·80	251·8	6·64	− 11·5	8·5	2·0
2·480	− 11·78	133·6	6·74	− 14·3	8·9	3·9
3·280	− 14·59	211·7	6·71	− 13·5	6·8	3·5

centrations of the ions is considerably greater in the protein solutions than in the dialysates, and that the protein must affect the mean activities of the ions of ammonium phosphates, although such measurements do not prove that the phosphates specifically are affected by the protein.

In addition to the measurements on ammonium phosphates, a number of experiments were made with a buffer containing 0·0533 mol. of Na_2HPO_4 *plus* 0·0133 mol. of KH_2PO_4, p_H 7·4 at 0°, which showed that the provisional estimate of $(E/C_v)_0=0·32$, given by Adair and Robinson [1930, 2], is too large. The value of $n_p=-25·4$ has been calculated from a more recent determination $(E/C_v)_0=0·24$. This value of 25·4 is larger than that obtained from 0·01 M ammonium phosphates at p_H 7·06 (17·1), but the difference is accounted for by the composition of the buffers.

Although the results obtained with serum-albumin and phosphate mixtures are consistent with formula (17) if the additional assumption is made that the phosphate is not completely ionised, they cannot be regarded as a proof of the formula on account of the lack of independent evidence.

Summary.

The sources of error in measurements of the membrane potentials of protein solutions have been investigated, and a method has been developed which can be applied to systems where the potentials are small and volumes of the solution available are restricted to 5 ml. or 1·5 ml.

The haemoglobin of sheep and ox, serum-albumin, acid haematin, kathaemoglobin and edestin have been equilibrated at 0° with dialysates of well-defined hydrogen ion concentration, with p_H values ranging from 2·0 to 7·8 and salt concentrations from 0·005 to 0·16 M, and it has been found that the observed relationship between the membrane potential E and the concentration C_v, expressed in g. protein per 100 ml. solvent, is a close approximation to a straight line when E is less than 2·0 millivolts.

The hypothesis is advanced that, from such measurements, it is possible to calculate the mean valence of the protein ions, symbolised n_p, by the formula $n_p=0·00425\,MJ\,(E/C_v)_0$, where M is the molecular weight of the protein and J the sum of the concentrations of the ions in the dialysate multiplied by the squares of their valences. $(E/C_v)_0$ is the limiting value of the ratio at $C_v=0$, determined by extrapolation. The hypothesis is consistent with measurements of the relationships between membrane potentials and protein concentrations and with additional evidence obtained from chemical analyses and osmotic pressure determinations on dilute solutions of edestin and on Congo red.

In the case of serum-albumin, the results obtained can be reconciled with the investigations of the distribution of ions on the assumption that the protein exists as a zwitterion and that some of the positive charges are neutralised by phosphate ions.

References.

Abramson (1933). *J. Gen. Physiol.* **16**, 593.
Adair (1925). *Proc. Roy. Soc. Lond.* A **108**, 627.
—— (1928). *Proc. Roy. Soc. Lond.* A **120**, 537.
—— (1929, 1). *J. Amer. Chem. Soc.* **51**, 696.
—— (1929, 2). *Proc. Roy. Soc. Lond.* A **126**, 16.
—— and Robinson (1930, 1). *Biochem. J.* **24**, 993.
—— —— (1930, 2). *Biochem. J.* **24**, 1864.
—— —— (1931). *J. Physiol.* **72**, 2 P.

Bayliss (1911). *Proc. Roy. Soc. Lond.* B **84**, 229.
Bell and Doisy (1920). *J. Biol. Chem.* **44**, 55.
Bjerrum (1924). *Z. physikal. Chem.* **110**, 656.
—— and Unmack (1929). *Kgl. Danske Vid. Selsk. Mat. Fys. Med.* (9), 1.
Brønsted (1929). *Z. physikal. Chem.* **143**, 301.
Clark (1928). The determination of hydrogen ions. (Baltimore.)
Cole (1933). Practical physiological chemistry. (Cambridge.)
Debye and Hückel (1923). *Physik. Z.* **24**, 185.
Donnan (1911). *Z. Electrochem.* **17**, 572.
—— and Allmand (1914). *J. Chem. Soc.* **105**, 1941.
—— and Guggenheim (1932). *Z. physikal. Chem.* **162**, 346.
—— and Harris (1911). *J. Chem. Soc.* **99**, 1554.
Ferguson, Van Lente and Hitchens (1932). *J. Amer. Chem. Soc.* **54**, 1279.
Guggenheim (1929). *J. Physical Chem.* **33**, 842.
—— (1930). *J. Amer. Chem. Soc.* **52**, 1315.
Hardy (1899). *J. Physiol.* **24**, 288.
—— (1905). *J. Physiol.* **33**, 251.
Hastings, Sendroy, Murray and Heidelberger (1924). *J. Biol. Chem.* **61**, 317.
Henriques (1928). *Biochem. Z.* **200**, 10.
—— (1929). *Ergeb. Physiol.* **28**, 625.
Hitchcock (1923). *J. Gen. Physiol.* **5**, 383.
Hückel (1925). *Kolloid Z.* **36**, 204.
Kameyama (1925). *Phil. Mag.* **50**, 849.
Kondo (1924). *Compt. Rend. Lab. Carlsberg*, **15**, No. 8.
Lamb and Larson (1920). *J. Amer. Chem. Soc.* **42**, 229.
Lewis, Brighton and Sebastian (1917). *J. Amer. Chem. Soc.* **39**, 2245.
—— and Randall (1923). Thermodynamics. (New York.)
Loeb (1922). Proteins and the theory of colloidal behaviour. (New York.)
McBain, Laing and Titley (1919). *J. Chem. Soc.* **115**, 1279.
Maclagan (1929). *Biochem. J.* **23**, 309.
Northrop and Kunitz (1926). *J. Gen. Physiol.* **9**, 351.
Pauli and Valkó (1933). Kolloid Chemie der Eiweisskörper. (Dresden.)
Rinde (1926). *Phil. Mag.* 1 (7), 32.
Sidgwick (1927). The electronic theory of valency. (Oxford.)
Sørensen (1917). *Compt. Rend. Lab. Carlsberg*, **13**, No. 5.
—— and Linderstrøm-Lang (1924). *Compt. Rend. Lab. Carlsberg*, **15**, No. 6.
—— and Sørensen (1933). *Compt. Rend. Lab. Carlsberg*, **19**, No. 11.
Stadie and Hawes (1928). *J. Biol. Chem.* **77**, 241.
—— and Sunderman (1931). *J. Biol. Chem.* **91**, 227.
Taylor, H. (1924). *Proc. Roy. Soc. Lond.* B **96**, 383.
Taylor, P. B. (1927). *J. Physical Chem.* **31**, 1478.
Tiselius (1930). *Nova Acta Reg. Soc. Sci. Upsaliensis, Ser.* IV, **7**, No. 4.
Van Slyke (1923). *J. Biol. Chem.* **58**, 523.
—— and Neill (1924). *J. Biol. Chem.* **61**, 523.
Vickery and Leavenworth (1928). *J. Biol. Chem.* **79**, 377.
Weber and Nachmannsohn (1929). *Biochem. Z.* **204**, 215.

XXX. THE ACIDS PRODUCED FROM SUGAR BY A *PENICILLIUM* PARASITIC UPON *ASPERGILLUS NIGER*.

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(*Received December 1st, 1933.*)

The mould whose behaviour on sugar solutions forms the subject of the present communication is a *Penicillium* originally isolated from an unsterilised cane sugar nutrient solution. Moulds believed to be of the same species have since been encountered contaminating *Aspergillus* cultures. The same or very similar species have also been found in nature accompanying *Aspergillus niger*. The association of the *Penicillium* with the latter mould is so intimate and its ultimate effect upon the *Aspergillus* so unfavourable that it may fairly be described as parasitic.

Thom [1930] states that members of the *P. rugulosum* series have frequently been found as parasites of other moulds in studies of the *Aspergilli*. Elsewhere Thom and Church [1926] refer a parasite of *A. niger* to the group *P. luteum-purpurogenum* close to *P. rugulosum*.

The present *Penicillium* bears conidia which resemble in size, shape and markings, and nearly in colour, those of *P. rugulosum* Thom. In some other respects, notably in its rapid and intense production of a diffusible red pigment in the mycelium, it resembles rather *P. purpurogenum* Stoll. On certain substrates, *e.g.* on malt agar, it shows a funiculose phase, forming ropes of red hyphae. On this medium there is often an early growth of pink or red pointed tufts of hyphae. Mature cultures on Czapek agar have herbage of a colour almost matched by "dull blackish green" of Ridgway, Plate XLI, m. 33, with here and there patches of yellow hyphae. The red pigment in the inferior surface and medium already referred to is very characteristic. Cultures have no marked odour. Colonies are not floccose. Sclerotia have not been observed. The penicillus is symmetrically biverticillate. The metulae, usually four in number, measure $11\mu \times 2{\cdot}5\mu$. The sterigmata $11\mu \times 2{\cdot}2\mu$. The conidial chains, which may be very long and densely packed, usually remain intact in fluid mounts. The conidia are elliptical ($3{\cdot}4\mu \times 2{\cdot}6\mu$), pigmented and have a roughened contour when ripe.

It is not proposed to attempt to identify the organism more closely than this, but to refer to it by the index letters under which it has been kept in culture, as *Penicillium* "*R.B.*"

In its action upon sugar solutions, *Penicillium* "*R.B.*" has been compared with type cultures of the other species named above. It is found to differ sharply from them by giving rise to an acid substance which is almost insoluble in water, but easily soluble in alcohol and other solvents specified later. Colourless crystals of this substance encrust the mycelium and glass in acid conditions. When chalk is present a soluble calcium compound forms in the solution. The explanation offered by Alsberg and Black [1913] for the distribution of mycophenolic acid according to the reaction of the medium probably applies here also.

As regards tolerance towards acidity *Penicillium* "*R.B.*" grows well on carbohydrate media over a large range of p_H and is tolerant of a high initial acidity in the medium. Thus, for example, it will grow well on sugar solutions in the presence of chalk and on similar sugar solutions having an initial p_H as low as 2·5. Nevertheless a certain acidity appears favourable for the germination of the conidia, and in the earlier experiments on chalk-containing media germination was quite erratic, frequently not taking place at all. On acidified media this trouble has never been experienced, and the difficulty with chalk was overcome by allowing germination to begin under acid conditions and then running the desired quantity of sterilised chalk suspension beneath the mould into the liquid. The conidia of many other moulds are known to germinate best in acid conditions [Webb, 1921]. The cultural device just mentioned may therefore have a more general interest.

The mould grows over a wide range of temperatures, with a growth-optimum probably at about 37°. It will grow at room temperature and 40°. Seed tubes kept over a year in cold store remained in perfectly good condition and gave rise to normal flask cultures.

The appearances of the cultures in the two cases where chalk is added after germination and where it is not added are very different. The biochemical changes and the fate of the sugar are also greatly affected as the following general description of the outstanding features at 28° in each case will show.

On chalk-containing sucrose medium, an opaque white mycelium forms comparatively rapidly. The inferior surface rapidly develops a blood-red colour which can be seen to diffuse into the solution. It begins first on those areas immediately over the chalk and can be hastened by disturbing the chalk from time to time. Sometimes the mould spores early, but will often remain free from spores to the end. Its surface then passes through various warm shades of colour—yellow, orange, rusty and red shades. Though the chalk is visibly disappearing there is little sign of any evolution of CO_2. The solution becomes more or less viscous. After some days white nodular crusts of calcium citrate separate out on the mycelium and the walls of the flask. Small quantities of calcium oxalate can also be demonstrated. After 10 days the solution may be run out and filtered from the calcium citrate and any remaining chalk. The filtrate gives a stronger calcium reaction than is accounted for by the dissolved calcium citrate. If it is acidified with HCl a cloud forms, often tardily, and on warming, coagulation and crystallisation take place. At this stage a characteristic aromatic rather pleasant smell is given off. When precautions are taken to decolorise the filtrate before these operations, these crude crystals are practically colourless.

On acidified sucrose medium the young mycelium is more frail and translucent and has a dangerous tendency to become "water-logged" and become submerged. Herrick and May [1928] record a similar observation with a variety of *P. purpurogenum* under certain conditions. *Penicillium* "*R.B.*" usually remains free from spores and shows more generous folding but produces no red colour. The solution is colourless, and in the early days becomes opalescent. It clears later, and small bunches of colourless crystals are found hanging from the mycelium or fallen to the bottom or adhering to the sides of the flask. From these may be obtained by purification and recrystallisation a substance which appears to be identical with "glauconic acid I" of Wijkman [1931].

The solution gives no detectable reaction for citric acid. The titratable acidity after the removal of the crystals is negligible. The crystals themselves show the same solubilities and behaviour as those derived from the calcium compound in the chalk cultures. If the inferior surface of the mycelium is touched with alkali, the bright red colour characteristic of the chalk-grown mycelium appears.

The substantial suppression under acid conditions of any accumulation of citric acid—an acid whose formation is known to be favoured by acid reactions in the case of other moulds [Currie, 1917; Butkewitsch, 1924]—and its accumulation when chalk is present is noteworthy. If oxalic acid is formed at all, it is only in traces. The presence of gluconic acid is not excluded.

Experimental.

Seed tubes for the inoculation of flask cultures have been grown on Czapek agar; malt-extract agar; and bread. Of the different temperatures used, 20°, 28° and 37°, 28° is probably the best for maintaining characteristics during subculture. 28° is also a satisfactory temperature for the incubation of flask cultures on sugar media.

(*a*) *Typical fermentation with* Penicillium "R.B." *on chalk-containing medium.*

Currie's solution, sucrose 150 g., NH_4NO_3 2·5 g., KH_2PO_4 1·0 g., $MgSO_4$, $7H_2O$ 0·25 g., HCl to p_H 3·4–3·5, water to 1000 ml. was used as a basis for culture solution. 250 ml. Erlenmeyer flasks containing 50 ml. of the solution were used. The initial p_H of the medium ensured sterility after 15–20 minutes' steaming at atmospheric pressure.

Five flasks inoculated from a bread culture of *Penicillium "R.B."* grown at 28° were incubated at 30–31°. After 16 hours' germination conidia had formed a delicate surface veil. 10 ml. of a sterilised chalk suspension were then run into each flask by pipette and the cultures returned to the incubator. After 10 days the cultures were sterilised and the liquors filtered. Washings from the mould and calcium citrate and the residual chalk were added to the filtrate. Analysis of a small sample of the filtrate gave a total dissolved calcium of 0·823 g. Rather more than the equivalent of hydrochloric acid was then added, and the fluid became turbid. On boiling, a quantity of crude crystals weighing 1·8 g. was deposited. This is 4·8 % of the original sugar in solution.

Numerous flask experiments on this medium have been made at various temperatures. The water-soluble calcium compound is produced more freely at higher temperatures, 28°–37°, than at room temperature, and 28° was chosen. Other experiments on the same medium were made to find what differences resulted from adding the chalk suspension successively later and later after the germination of spores. Addition 12–18 hrs. after the beginning of incubation at 28° was found to give satisfactory types of culture. Other tests were made to detect differences due to agitating the chalk during incubation, without any decisive result—probably owing to the small depth of the solution. Among early fermentations many were designed to overcome the difficulty of irregular germination already mentioned.

The crude insoluble acid from most of these small tests was saved and bulked for purification later.

The solid calcium compounds were dissolved in excess of HCl, filtered and re-neutralised in the cold. A small precipitate, not wholly soluble on addition of acetic acid, formed. This was removed, and from it were obtained characteristic crystals of calcium oxalate. The calcium citrate in the solution was precipitated by boiling, filtered off and washed with boiling water. The dry weight of calcium citrate was 6·4 g. equivalent to 4·7 g. of citric acid; *i.e.* 12·5 % of the original sugar in solution.

Residual sugar was not estimated.

(*b*) *Typical fermentation with* Penicillium "R.B." *on acid medium.*

To examine the behaviour of *Penicillium "R.B."* in absence of chalk a similar medium of the following composition was prepared and steamed for 20 mins. at atmospheric pressure: sucrose 150 g., NH_4NO_3 2·25 g., KH_2PO_4 0·875 g., $MgSO_4, 7H_2O$ 0·225 g., HCl 4·5 ml. of $N/5$, water to 1000 ml. Flasks, quantities of liquid and incubation temperature as before.

The mycelium had a slight peppering of olive-green spores interspersed with tufts of pale sulphur-yellow hyphae; it became only pale yellow on the under surface with traces of red at a few points on the rim in contact with the glass. After 11 days when the cultures were run out numerous small colourless crystals covered the under surface and the bottom of the flask.

Fermentation liquor and loose crystals were run out into a beaker, the bulk of those adhering to the mycelium were detached in running water and added to those in the beaker and all washed by decantation. They were filtered, washed with cold water and dried at 100°. The average weight crystallised from each flask was 0·125 g., 1·66 % of original sugar supplied. Melting-point of the crude crystals insoluble in water was between 193 and 197°. The average weight of dry mycelium from each flask was 0·28 g. The increase in the titratable acidity of the liquor was negligible. Qualitative tests for oxalic acid and citric acid were both negative. As before the sugar of the medium was not exhausted.

Many other small flask fermentations in absence of chalk have been carried out, varying the nutrients in solution, the temperature of incubation, concentration of sugar, p_H of solution and other factors. Many of these produced crude crystals. The yields reported are not large, but other experiments in progress indicate the possibility of considerably increasing the yield.

One solution which failed to yield any crystals was the Czapek-Dox solution. In this case, the cultures spored early and the fluid developed the red colour. The sugar in this solution is glucose, but that it was not the change from sucrose to glucose which caused the difference was proved by other tests.

Purification of the water-insoluble crystals. A quantity of crude crystals obtained as described above was dissolved in an excess of N NaOH. The solution was clarified with norite and filtered. The filtrate was acidified with strong HCl and an almost pure white product obtained, giving, after washing and drying at 100°, over 37 g. The melting-point of this material was not sharp, however, but ranged from 196 to 199°.

A part of this was submitted to recrystallisation from glacial acetic acid. This gave excellent crystals, the presumably purest specimens of which melted at 201–202°.

Properties. The substance is practically insoluble in cold water and only slightly soluble in hot water. It is insoluble in light petroleum. It dissolves in alcohol, acetone, ether, benzene, chloroform, pyridine and glacial acetic acid, from which it crystallises with ease. It dissolves in sodium carbonate solution with the evolution of CO_2, only gradually in dilute solutions of NaOH and may be reprecipitated with acid as described. It may also be recovered crystalline by adding water to a solution in alcohol and warming. It does not reduce Fehling's solution or ammoniacal silver nitrate. The bromine absorption test is negative, and it gives no coloration with $FeCl_3$. In concentrated H_2SO_4 it blackens on heating. It burns with a smoky flame.

Titration. The substance was dissolved in ethyl alcohol and titrated with $N/10$ NaOH in presence of phenolphthalein. In the cold there is no very definite

end-point; the alkali is only neutralised slowly. More soda is taken up on heating.

		Equivalent
Cold	0·0774 g. required 5·7 ml. $N/10$ NaOH	—
Hot	0·0774 g. required 8·55 ,, ,,	90·5
	0·1842 g. required 20·3 ,, ,,	90·7
	0·1591 g. required 18·2 ,, ,,	87·4

(Theoretical for $C_{18}H_{20}O_7$ titrating as a tetrabasic acid, 87·0.)

A search of the literature at the time failed to disclose any compound with the above properties produced by a mould. Before an opportunity occurred for a fuller chemical investigation of the new acid, Wijkman's work on the production of "glauconic acid I and II" by a *P. glaucum* appeared [Wijkman, 1931].

Her experiments were made on a sucrose solution containing very small quantities of mineral salts and without chalk present. In these circumstances she obtained insoluble acids crystallising out during the fermentation and from the crude mixture separated two pure substances, the properties of one of which, glauconic acid I, agree well with those of the acid produced by *Penicillium* "*R.B.*" Glauconic acid I is given the formula $C_{18}H_{20}O_7$, and has a melting-point of 202°.

A thorough chemical examination of the metabolic products of the *Penicillium* "*R.B.*" fermentation has still to be made, but the properties of the insoluble acid already described have since been compared with those described by Wijkman.

Mol. wt. 386, 380; micro-analysis (Schoeller) C, 62·07; H, 5·75 %; calculated for $C_{18}H_{20}O_7$; mol. wt. 348; C, 62·04; H, 5·79 %.

By acting on the acid dissolved in pyridine with acetic anhydride a crystalline derivative has been prepared whose melting-point closely approaches the value 175° given for the corresponding derivative of glauconic acid I. Micro-analysis C, 61·52; H, 5·64 %; calculated for mono-acetyl derivative C, 61·61; H, 5·68 %.

Heated above its melting-point, the original acid gives off a very fragrant vapour as the substance decomposes, as does glauconic acid I.

A substance corresponding to glauconic acid II has not been recovered, but the mother-liquors from the recrystallisation have not been investigated, so that the presence of this cannot be excluded.

The experiments already described were all made with sucrose. Fermentations were made upon 15 % solutions of glucose and fructose and in all cases crystals of the same appearance, giving the same qualitative tests as those obtained with sucrose, resulted. When chalk is present citric acid is also formed in quantities of the same order as with sucrose. Glycerol has also been tried, and the striking feature of the glycerol fermentation is that the solution instead of remaining colourless becomes a deep yellow.

Summary.

A *Penicillium* parasitic on *Aspergillus niger* is described. Its action upon sucrose solutions has been examined.

In the presence of chalk, considerable quantities of calcium citrate and small quantities of calcium oxalate are formed; also the soluble calcium salt of an acid insoluble in water, but soluble in alcohol.

In acid solutions the insoluble acid is formed encrusting the mould and separating on the vessel, but detectable quantities of citric and oxalic acids are not formed.

The insoluble acid also appears when sucrose is replaced in the medium by glucose or fructose. Citric acid is also formed from glucose and fructose when chalk is present.

The insoluble acid is probably to be identified with "glauconic acid I" of Wijkman, originally found in cultures of a *P. glaucum*.

REFERENCES.

Alsberg and Black (1913). *U.S. Dept. of Agriculture, Bureau of Plant Industry Bulletin* No. 270, 46.
Butkewitsch (1924). *Biochem. Z.* **154**, 177.
Currie (1917). *J. Biol. Chem.* **31**, 15.
Herrick and May (1928). *J. Biol. Chem.* **77**, 185.
Thom (1930). The *Penicillia*. (Williams and Wilkins.)
—— and Church (1926). The *Aspergilli*. (Williams and Wilkins.)
Webb (1921). *Ann. Missouri Bot. Garden*, **8**, 283.
Wijkman (1931). *Liebig's Ann.* **485**, 61.

XXXI. PHENYL *ISO*CYANATE PROTEIN DERIVATIVES AND THEIR IMMUNOLOGICAL PROPERTIES.

III. THE AMINO-ACID DERIVATIVES AND SEROLOGICAL INHIBITION TESTS.

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In previous papers [Hopkins and Wormall, 1933, 1, 2] the chemical and immunological properties of phenylcarbamido-proteins have been recorded. It is to be presumed that in these compounds the $C_6H_5.NH.CO$-residue is attached to the free amino-groups of the lysine molecules. In view of the fact that serological inhibition tests have considerable value in the study of the constitution of chemically altered proteins, it was decided to make a more extensive study of the phenylcarbamido compounds. The phenyl- and *p*-bromophenylcarbamido-acids derived from many of the common amino-acids and similar compounds from α- and ε-amino-*n*-hexoic acid have therefore been prepared and have been used in precipitin and complement-fixation inhibition tests in the hope that more knowledge might be gained about the structure of proteins and the nature of the reaction between native proteins and phenyl *iso*cyanates. In particular it was hoped that these tests would give more information about the free basic groups of the intact protein molecule.

Preparation of the phenylcarbamido-acids.

These compounds were prepared by the action of phenyl *iso*cyanate (1·4 mol.) on the amino-acid (1 mol.) dissolved in water containing NaOH (1 mol.). Double quantities of NaOH were used for the preparation of the aspartic acid, cystine and tyrosine compounds and three times the normal quantity when lysine dihydrochloride was used. The amount of phenyl *iso*cyanate was doubled for the reactions with lysine, cystine and tyrosine, since the first two contain two amino-groups per molecule and tyrosine has a hydroxyl group which under certain circumstances might react with the *iso*cyanate.

The mixture of the *iso*cyanate and the solution of the sodium salt of the amino-acid was well stirred at room temperature (15–18°) for 1 hour, 0·5 *N* NaOH being added where necessary to maintain a slight alkalinity. The solution was then filtered, and the phenylcarbamido-acid was precipitated from the filtrate by the addition of dilute HCl, with cooling where necessary. Most of these compounds were recrystallised twice from water or dilute alcohol and dried *in vacuo* over $CaCl_2$. It was not found possible to recrystallise the lysine compound, and it was purified by reprecipitation. The yield of the recrystallised product was usually quite satisfactory. The melting-points recorded are all uncorrected.

The phenylcarbamido-acids used were all known compounds except those obtained from α- and ε-amino-*n*-hexoic acids which had M.P. 140–141° and 126–127° respectively.

Preparation of the p-*bromophenylcarbamido-acids.*

These compounds were prepared by the action of *p*-bromophenyl *iso*cyanate (1·4 mol.) dissolved in ether on the amino-acid (1 mol.) in slightly alkaline solution (1, 2 or 3 mol. of NaOH according to the amino-acid used, as in the case of the phenyl *iso*cyanate compounds).

The reaction mixture was cooled in ice and shaken for about 1 hour, more NaOH being added as necessary to keep the solution alkaline. The solution was then filtered and the *p*-bromophenylcarbamido-acid precipitated as before and recrystallised twice. It was not found possible to recrystallise the lysine and cystine compounds. Bromine estimations (Carius) and melting-point determinations were carried out on all the products. The results are tabulated below.

Properties of the p-*bromophenylcarbamido-acids.*

Amino-acid	M.P. of product	% Br Found	% Br Calc.	Crystalline form
Glycine	Decomposes 200–210° without melting	29·2	29·3 (for $C_9H_9O_3N_2Br$)	Flat prisms
Alanine	181–183° (decomp.)	27·7	27·9 (for $C_{10}H_{11}O_3N_2Br$)	Flat prisms
Leucine	162–164° ,,	24·3	24·3 (for $C_{13}H_{17}O_3N_2Br$)	Prisms
α-Amino-*n*-hexoic acid	181–182° ,,	24·6	24·3 (for $C_{13}H_{17}O_3N_2Br$)	Prisms
ε-Amino-*n*-hexoic acid	170–172° ,,	24·0	24·3 (for $C_{13}H_{17}O_3N_2Br$)	Flat prisms
Lysine	176–177° ,,	28·5	29·5 (for $C_{20}H_{22}O_4N_4Br_2$)	Non-crystalline
Cystine	174–175° ,,	24·2	25·1 (for $C_{20}H_{20}O_6N_4S_2Br_2$)	Non-crystalline
Aspartic acid	175–176° ,,	24·5	24·2 (for $C_{11}H_{11}O_5N_2Br$)	Prismatic plates
Tyrosine	197–199° ,,	21·0	21·1 (for $C_{16}H_{15}O_4N_2Br$)	Flat prisms

The bromine values recorded in the above Table indicate that the normal reaction between *p*-bromophenyl *iso*cyanate and the amino-group of the amino-acid occurs with all the amino-acids tested. In the cases of cystine and lysine, the calculations are based on the introduction of two of these groups into each molecule of the amino-acid. The Br values obtained with these preparations were significantly lower than the calculated values, and it appears probable that these preparations consisted mainly of the disubstituted compounds with smaller amounts of the monosubstituted compound or compounds.

Inhibition tests.

Landsteiner [1920] first showed that some simple substances could specifically inhibit the formation of a precipitate when certain antisera were added to corresponding antigens. In these tests a neutral solution of the substance, which is to be tested for inhibitory power, is mixed with the antigen solution. To this

mixture is then added an antiserum which normally gives a marked precipitate with the antigen. If no precipitate is produced (*i.e.* complete inhibition), and if the substance does not inhibit any other type of precipitin reaction (*e.g.* that between horse-serum and an antiserum against horse-serum), specific union between antibody and inhibiting substance has occurred. This substance might therefore be said to have groupings similar to the antigenically prominent groupings of the antigen. The value of such tests will be discussed later in this paper.

Precipitin inhibition tests. Precipitin tests were carried out under the conditions described previously [Hopkins and Wormall, 1933, 1, 2]. In the inhibition tests, 0·05 ml. of the antigen solution (0·025 % phenylcarbamido-horse-serum-proteins or more dilute solutions of phenylcarbamido-gelatin) was added to 0·20 ml. of a carefully neutralised solution of the inhibitory substance (0·01, 0·005 or 0·0025 *M*). To this mixture was added 0·10 ml. of the antiserum (antiserum against phenylcarbamido-horse-serum-globulin) and the degree of precipitin formation noted immediately and after 5 mins., $\frac{1}{2}$ hr. and 1 hr. These precipitin reactions were carried out at 37° except where the phenylcarbamido-gelatin compounds were used, these last-named compounds giving more pronounced precipitates at room temperature [Hopkins and Wormall, 1933, 2]. The results of the precipitin reactions are recorded as follows (in increasing degrees of precipitation): – (no reaction), f. tr. (faint trace), tr. (trace), ±, +, +±, ++, *etc.*

Complement fixation inhibition tests. To a series of tubes each containing 0·5 ml. of diluted antigen solution, the range comprising 1:20, 1:60, 1:180 down to 1:1,180,980 of a 5 % antigen solution, were added 0·10 ml. of a neutral 0·01 *M* solution of the inhibiting substance (in 0·9 % NaCl solution), 0·10 ml. of 1:5 guinea-pig-serum and 0·05 ml. of the antiserum. After these mixtures had been allowed to stand for $\frac{3}{4}$ to 1 hr. at room temperature, 0·5 ml. of a 4 % suspension of sensitised ox red cells (in 0·9 % NaCl) was added to each tube. The tubes were immersed in a water-bath at 37° and the extent of haemolysis noted at intervals. In many experiments similar series of tests were made with larger or smaller amounts of guinea-pig complement, the results being essentially the same as those with the amount recorded above.

Antigens and antisera.

The preparation of the antigens and antisera used for this investigation has been described in previous papers [Hopkins and Wormall, 1933, 1, 2]. The antigen concentrations (1:20, 1:100, *etc.*) refer to dilutions of a 5 % solution.

Experimental.

The phenyl- and *p*-bromophenyl-carbamido-acids were tested for the power to inhibit the reaction between phenylcarbamido-proteins and corresponding antisera. The antisera used were those against phenylcarbamido-horse-serum-proteins, whereas the antigen was some other phenylcarbamido-protein, either phenylcarbamido-chicken-serum-proteins or phenylcarbamido-gelatin. The last-named antigen proved of special value for this purpose, since with the antisera mentioned it gives marked precipitates in very high dilutions, and in these inhibition tests it is of advantage to reduce the amount of antigen to the minimum necessary to give a good precipitin reaction. In this way it is possible to reduce the amount of antigen competing with the inhibiting substance, and any

inhibitory action by the latter is usually more definite with the lower concentration of antigen.

These inhibition tests have been made with several concentrations of the antigen and antisera and with different amounts of the substances tested for inhibitory power. The last-named were usually used in 0·01 *M* solution in 0·9 % NaCl, this being about the maximum solubility for some of the substances. The results of a typical experiment are given in Table I, this Table giving readings with two concentrations of antigen and two concentrations of the inhibiting substances.

Table I. *Precipitin inhibition tests.*

Antigen: Phenylcarbamido-chicken-serum-proteins.
Immune-serum: Antiphenylcarbamido-horse-serum-globulin (No. 51).
Temperature: 37°.

	Antigen solution			
	1 : 20	1 : 20	1 : 100	
	Solution of inhibiting substance			
			0·01 *M*	
Inhibiting substance	0·01 *M*	0·005 *M*		
NaCl	+ ±	+ ±	+ ±	+ ±
Glycine	+ ±	+ ±	+ ±	+ ±
Alanine	+ ±	+ ±	+ ±	+ ±
α-Amino-*n*-hexoic acid	+ ±	+ ±	+ ±	+ ±
Lysine	+ ±	+ ±	+ ±	+ ±
Phenylcarbamido-acetic acid	+	+	tr.	±
α-Phenylcarbamidopropionic acid	+	+	tr.	±
α-Phenylcarbamido-*n*-hexoic acid	+	+	tr.	±
αε-Diphenylcarbamido-*n*-hexoic acid	±	±	–	f.tr.
αε-Diphenylcarbamido-*n*-hexoic acid*	±	±	–	f.tr.
p-Bromophenylcarbamido-acetic acid	+ ±	+ ±	tr.	±
Readings after:	30 mins.	30 mins.	5 mins.	30 mins.

* Tests with one-half of the corresponding 0·01 or 0·005 *M* solutions.

From this table it will be seen that the phenylcarbamido-acids have a marked inhibitory influence on the precipitin reaction between phenylcarbamido-proteins and the corresponding antisera, and that the most pronounced effect is exhibited by the lysine derivative. Preliminary experiments of this nature were used in support of the view that the reaction between phenyl *iso*cyanate and proteins involves the free amino-groups of the lysine molecules of the protein [Hopkins and Wormall, 1933, 1]. Since lysine has two NH_2-groups it seems possible that the corresponding phenylcarbamido-acid might be most efficient as an inhibitory agent because it contains two phenylcarbamido-groupings per molecule, but this explanation was soon excluded, for the lysine compound always gives much better inhibition than does twice the equivalent amount of the other α-phenylcarbamido-acids. In the experiments described in this paper, tests with one-half the equivalent amounts of the lysine compounds were always made, the results being identical with those obtained with the higher concentrations of these compounds.

The results given in Table I demonstrate the advantage of using as little antigen as possible for these inhibition tests, although it is necessary, of course, to obtain a good precipitate in the control tests, and they indicate also the added information which is gained by taking readings at various intervals of time. The results with *p*-bromophenylcarbamido-acetic acid show that this compound has some inhibitory action on the phenylcarbamido-precipitin reaction, but that the effect is less marked than that produced by phenylcarbamido-acetic acid.

One of the main objects of the work described in this paper was to obtain as much information as possible, using immunological methods, about the free basic groups of the protein molecule. For this purpose α- and ε-amino-*n*-hexoic acids and their phenyl- and *p*-bromophenyl-carbamido-derivatives were prepared and these compounds and the corresponding lysine compounds compared

Table II. *Precipitin inhibition tests.*

Immune serum: Antiphenylcarbamido-horse-serum-globulin (No. 51).
Temperature: 16°. (Readings after 30 mins.).

Inhibiting substance		Antigen: Phenylcarbamido-gelatin (1 : 500)		Antigen: *p*-Bromophenylcarbamido-gelatin (1 : 500)
NaCl		+ ±	+ ±	+
Alanine (lysine, or α- or ε-amino-*n*-hexoic acid)		+ ±	+ ±	+
Phenylcarbamido-acid corresponding to	alanine	tr.	+	tr.
	lysine	−	−	−
	α-amino-*n*-hexoic acid	tr.	+	tr.
	ε-amino-*n*-hexoic acid	−	−	−
Concentration of inhibiting substance:		0·01 *M*	0·0025 *M*	0·01 *M*

Note. None of the amino-acids used in this investigation, or the corresponding phenylcarbamido-acids, had any inhibitory influence in these concentrations (0·01 *M* and more dilute solutions) on the precipitin reaction between horse-serum-globulin and an antiserum to horse-serum-globulin.

Table III. *Precipitin inhibition tests.*

Antigen: Phenylcarbamido-chicken-serum-proteins (1 : 100) and phenylcarbamido-gelatin (1 : 500).
Inhibiting substance: Neutralised 0·01 *M* solutions (in 0·9 % NaCl).
Antiserum: Antiphenylcarbamido-horse-serum-globulin (No. 94).
Temperature: 37° for the chicken-serum compound and 16° with phenylcarbamido-gelatin as antigen.

Inhibiting substance		Antigen: Phenylcarbamido-chicken-serum-proteins		Antigen: Phenylcarbamido-gelatin	
NaCl		+ +	+ +	+	+
Cystine (tyrosine, leucine, aspartic acid, α- and ε-amino-*n*-hexoic acids and lysine)		+ +	+ +	+	+
Phenylcarbamido-acid corresponding to	cystine	−	−	−	−
	tyrosine	tr.	tr.	−	−
	leucine	tr.	tr.	−	−
	aspartic acid	+	+	±	±
	α-amino-*n*-hexoic acid	−	−	−	−
	ε-amino-*n*-hexoic acid	−	−	−	−
	lysine	−	−	−	−
	lysine*	−	−	−	−
p-Bromophenylcarbamido-acid corresponding to	cystine	tr.	±	−	f.tr.
	tyrosine	tr.	±	f.tr.	f.tr.
	leucine	±	±	−	−
	aspartic acid	±	±	f.tr.	f.tr.
	α-amino-*n*-hexoic acid	±	±	−	−
	ε-amino-*n*-hexoic acid	−	−	−	−
Readings after:		½ hr.	1 hr.	½ hr.	1 hr.

* *Cf.* Note to Table I.

Note. Smaller amounts of the phenylcarbamido-acids derived from cystine, tyrosine, leucine, aspartic acid and α-amino-*n*-hexoic acid gave slight inhibition only, whereas with the compounds from lysine and ε-amino-*n*-hexoic acid the inhibition was still complete.

as inhibitors of the phenylcarbamido-precipitin reaction. From the results of a typical experiment of this nature (*cf.* Table II), it appears that of these two monoamino-*n*-hexoic acids the ϵ-compound is the one which most resembles the lysine compound in its inhibitory power.

The results recorded in Table III are similar to those in Tables I and II. They show that all the phenylcarbamido-acids tested have a marked inhibitory effect on the precipitin reaction between phenylcarbamido-proteins and corresponding antisera, whereas the amino-acids themselves are inactive. The phenylcarbamido-acid derived from aspartic acid gave moderate inhibition only when compared with equivalent amounts of the corresponding compounds from glycine, cystine, *etc.*, but this is probably due to the fact that the preparation obtained by the action of phenyl *iso*cyanate on aspartic acid is not very pure, and the recrystallised material represents a mixture of the free acid and the hydantoin [Paal and Zitelmann, 1903]. Thus all the phenylcarbamido-acids with the exception of the aspartic acid derivative gave complete or almost complete inhibition in the concentration used in the experiment recorded in Table III. With more dilute solutions of the inhibiting substances, the lysine and ϵ-amino-*n*-hexoic acid derivatives showed more marked inhibitory power than did those of the other amino-acids, as in the experiments recorded in Table II.

Table III also gives the results of tests with the *p*-bromophenylcarbamido-acids. These compounds, although not quite as effective as the corresponding phenyl compounds, possess a marked inhibitory capacity. The lysine derivative could not be used in these tests on account of its very slight solubility in water, but of the other compounds tested, the ϵ-amino-*n*-hexoic acid derivative was most effective. The introduction of bromine into the benzene ring of the phenylcarbamido-acids thus reduces somewhat but does not destroy the capacity of these substances to react with the antibodies to phenylcarbamido-proteins. The phenylcarbamido-acids are able similarly to inhibit the precipitin reaction between *p*-bromophenylcarbamido-gelatin (or the corresponding horse-serum compound) and antibodies to phenylcarbamido-proteins.

In Table IV a typical result is given showing the influence of some of the phenylcarbamido-acids on complement fixation tests, the other derivatives not

Table IV. *Complement fixation tests* (*inhibition tests*).

	Inhibiting substance										Phenylcarbamido-acid corresponding to							
	NaCl		Glycine		α-Amino-*n*-hexoic acid		ϵ-Amino-*n*-hexoic acid		Lysine		Glycine		α-Amino-*n*-hexoic acid		ϵ-Amino-*n*-hexoic acid		Lysine	
Antigen dilution																		
1 : 20	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
1 : 60	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
1 : 180	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
1 : 540	–	2	2	4	3	4	4	4	4	4	4	4	4	4	4	4	4	4
1 : 1620	–	–	–	–	–	–	–	1	–	–	3	4	4	4	4	4	4	4
1 : 4,860	–	–	–	–	–	–	–	–	–	–	2	3	3	4	4	4	4	4
1 : 14,580	–	–	–	–	–	–	–	–	–	–	–	3	–	3	3	4	4	4
1 : 43,740	–	–	–	–	–	–	–	–	–	–	3	4	1	3	4	4	4	4
1 : 131,220	–	1	–	–	–	–	–	–	–	–	3	4	3	4	4	4	4	4
1 : 393,660	2	4	–	–	–	–	–	–	–	–	4	4	3	4	4	4	4	4
1 : 1,180,980	4	4	–	1	–	1	–	1	–	1	4	4	4	4	4	4	4	4
Control (NaCl)	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Reading after (hr.)	¼	½	¼	½	¼	½	¼	½	¼	½	¼	½	¼	½	¼	½	¼	½

In the above table, 4 represents complete haemolysis, – no haemolysis, and 3, 2 and 1 intermediate degrees of haemolysis.
Phenylcarbamido-gelatin was the antigen used for these tests and the antiserum was one against phenylcarbamido-horse-serum-globulin.

quoted in this Table giving results similar to those with that prepared from glycine. Thus, these compounds are capable of effecting marked inhibition of the fixation of complement by mixtures of phenylcarbamido-gelatin and antisera to phenylcarbamido-proteins. This other type of inhibition test, involving inhibition of complement fixation, appears to give even more conclusive results than do the corresponding precipitin inhibition tests, and they confirm the finding that the phenylcarbamido-acids from lysine and ϵ-amino-*n*-hexoic acid are the most active of the amino-acid derivatives tested. In these complement fixation tests, as with the corresponding precipitin inhibition tests, experiments were made repeatedly to show that the inhibitory action of the phenylcarbamido-acids is specific, and that these compounds do not inhibit the interaction (in complement fixation and precipitin tests) between other antigens and their antibodies.

Discussion.

Specific inhibition of the precipitin reaction by simple substances was first described by Landsteiner [1920], and since then this reaction has been used with much success by Landsteiner and van der Scheer [1928; 1931; 1932, 1, 2] and by other authors. Specific inhibition is obtained when the added substance contains a grouping identical with, or related to, that which is antigenically active in the antigen. Thus, *p*-amino- and *p*-bromo-benzoic acids inhibit the precipitin reaction between *p*-aminobenzoic acid-azo-protein and its antibody, but much less inhibition is exerted by *o*-amino- or *o*-bromo-benzoic acid [Landsteiner, 1920]. In a similar manner, the reaction between azo-proteins formed by the coupling of a protein with diazotised aminobenzoyl-dipeptides (*e.g.* *p*-aminobenzoylglycylglycine) and antibodies to these compound proteins is specifically inhibited by the corresponding *p*-amino- or *p*-nitro-benzoyl-dipeptide [Landsteiner and van der Scheer, 1932, 1]. In many instances therefore it has been shown that simple substances can unite with antibodies and prevent, partially or completely, the reaction with the homologous antigen, and such tests are very helpful in studies on the interaction between antigen and antibody and in investigations concerned with immunological specificity. Such tests may also be used for more strictly chemical purposes. Thus, the inhibition of the reaction between iodinated proteins and their antisera by 3 : 5-dihalogenated tyrosine furnished very strong evidence in support of the view that the method of iodination used resulted mainly in the introduction of two iodine atoms into the 3 : 5-positions of each tyrosine grouping [Wormall, 1930].

From the investigations referred to above it will be seen that these inhibition tests can reveal much about the nature of groupings in chemically altered proteins, or proteins to which new groupings have been attached, and it appears highly probable that they will help considerably in attempts to learn more about the intact protein molecule. The tests do not involve any drastic treatment of the material to be tested, and they also allow the recognition of substances which cannot readily be isolated in the pure condition. Thus "inert" substances do not interfere with the inhibitory action of other substances, *e.g.* the specific inhibition of the phenylcarbamido-precipitin reaction by phenylcarbamido-acids, described in this paper, is not affected by the presence of unchanged amino-acids, *etc.* The tests can readily be used, therefore, to detect the presence of certain substances in a fairly complex mixture. They can also be used, in addition to the ordinary precipitin tests, in studies on spatial configuration [Landsteiner and van der Scheer, 1932, 3].

The work described in this paper has been concerned mainly with the free amino-groups of the protein molecule. Phenyl *iso*cyanate readily reacts with

peptones as was shown by Raper [1907], who first described these compounds, and a similar reaction occurs with native proteins [Hopkins and Wormall, 1933, 1, 2]. The reaction with native proteins will obviously be more restricted, since the number of free amino-groups in the molecule of a protein of this type is usually rather small, and with a protein like zein, which is lacking in these basic groups, no reaction occurs. In the phenylcarbamido-proteins therefore the new groups appear to be attached to the free amino-groups only, and evidence of a chemical and immunological nature was put forward in support of the view that the main new grouping in these protein compounds is attached to the ϵ-amino-group of lysine [Hopkins and Wormall, 1933, 1]. Lysine however is a diamino-acid ($\alpha\epsilon$-diamino-*n*-hexoic acid), and it would be very helpful if we could determine whether the new groupings are attached to the α- or the ϵ- or both the α- and ϵ- amino-groups of the lysine molecules: in other words, which amino-groups are free in the intact protein. The chemical evidence available suggests that the free grouping is the ϵ-amino-group [*cf.* Van Slyke and Birchard, 1913; Edlbacher, 1921; Goldschmidt and Kinsky, 1929; Kapfhammer, 1930; Abderhalden and Schweitzer, 1931].

This view about the free amino-groups of the intact protein molecule receives very strong support from the immunological evidence presented in this paper. Inhibition tests (precipitin and complement fixation) show that $\alpha\epsilon$-diphenylcarbamido-*n*-hexoic acid combines very readily with antibodies to phenylcarbamido-proteins, and that similar phenylcarbamido-acids derived from the common amino-acids (glycine, cystine, tyrosine, *etc.*), although they are quite active in this respect, do not show by any means the same degree of activity as the lysine derivative. Furthermore, a comparison of the phenylcarbamido-acids from α- and ϵ-amino-*n*-hexoic acids has demonstrated that the latter behaves like the lysine derivative and the former like those of the other amino-acids. Thus the configuration of ϵ-phenylcarbamido-*n*-hexoic acid shows most resemblance to that configuration in phenylcarbamido-proteins which reacts with phenylcarbamido-antibodies, which leads one to the conclusion that a large number of the ϵ-amino-groups of the lysine components of the protein are free. The marked difference between the inhibitory powers of the lysine and the ϵ-amino-*n*-hexoic acid derivatives on the one hand, and derivatives of the other amino-acids on the other, suggests that the majority, if not all, of the ϵ-amino-groups of the lysine are free, but this conclusion must await further experimental evidence. Although this particular problem might not be capable of final solution by serological tests similar to those used in this investigation, sufficient has been said here to indicate the value of such tests in studies on the chemistry of proteins.

Summary.

1. Phenyl and *p*-bromophenyl *iso*cyanate derivatives of several amino-acids have been prepared, including those of α- and ϵ-amino-*n*-hexoic acids.

2. Inhibition by these phenylcarbamido-acids of the precipitin reaction between phenylcarbamido-proteins and the corresponding antisera has been studied. Similar inhibition of the complement fixation reactions has been obtained.

3. The phenylcarbamido-acids from lysine and ϵ-amino-*n*-hexoic acid give complete inhibition, whereas equivalent amounts of the derivatives of α-amino-*n*-hexoic acid, leucine, alanine, tyrosine, *etc.*, give moderate inhibition only.

4. These serological inhibition tests offer, therefore, strong support for the view that the free amino-groups of the intact protein molecule are the ϵ-amino-groups of the lysine molecules.

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REFERENCES.

Abderhalden and Schweitzer (1931). *Fermentforsch.* **12**, 350.
Edlbacher (1921). *Z. physiol. Chem.* **112**, 80; **116**, 228.
Goldschmidt and Kinsky (1929). *Z. physiol. Chem.* **183**, 244.
Hopkins and Wormall (1933, 1). *Biochem. J.* **27**, 740.
—— —— (1933, 2). *Biochem. J.* **27**, 1706.
Kapfhammer (1930). *Z. physiol. Chem.* **191**, 112.
Landsteiner (1920). *Biochem. Z.* **104**, 280.
—— and van der Scheer (1928). *J. Exp. Med.* **48**, 315.
—— —— (1931). *J. Exp. Med.* **54**, 295.
—— —— (1932, 1). *J. Exp. Med.* **55**, 781.
—— —— (1932, 2). *Proc. Soc. Exp. Biol. Med.* **29**, 747; *J. Exp. Med.* **56**, 399.
—— —— (1932, 3). *Proc. Soc. Exp. Biol. Med.* **29**, 1261.
Paal and Zitelmann (1903). *Ber. deutsch. chem. Ges.* **36**, 3337.
Raper (1907). *Hofmeisters Beiträge*, **9**, 168.
Van Slyke and Birchard (1913). *J. Biol. Chem.* **16**, 539.
Wormall (1930). *J. Exp. Med.* **51**, 295.

XXXII. STUDIES ON XANTHINE OXIDASE.
XI. XANTHINE OXIDASE AND LACTOFLAVINE.

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Until recently the view was held by many that molecular oxygen cannot react directly with the reducing systems of the cell but requires the intermediation of catalytic iron. Experiments on oxidase systems extracted from cells seemed to support this view. The succinic oxidase of muscle, for instance, as shown by Thunberg [1917] and others, will reduce methylene blue or oxygen in presence of succinate, but if a small amount of HCN is added all power of reducing oxygen is lost, while methylene blue is still reduced with the same velocity as before. This was interpreted as being due to the inactivation of the iron compound acting as an intermediary between the activated substrate and molecular oxygen.

The xanthine oxidase of milk and liver was however shown by Dixon and Thurlow [1925] to be unaffected by HCN, both with respect to methylene blue reduction and oxygen uptake. This system was therefore assumed to act in accordance with Wieland's theory of oxidation, in which molecular oxygen acts as a direct acceptor of the hydrogen of the substrate molecule.

The work of Warburg and Christian [1932] has shown the existence of another type of compound in cells which functions as an intermediary between the reducing systems and oxygen. This is a yellow pigment which does not contain iron or any other metal. It is rapidly reducible by the hexosemonophosphate dehydrogenase system isolated from several kinds of cells and is then reoxidised by molecular oxygen with the formation of H_2O_2; it can function catalytically even in the presence of HCN.

The recent researches of Kuhn *et al.* [1933] and Stern [1933] have shown that yellow pigments of this type, now known as flavines, occur in both milk and liver, and the possibility therefore arose that in the xanthine oxidase system prepared from these sources the oxygen reacted directly with the leuco-form of the yellow pigment and not directly with the substrate. The formation of H_2O_2 observed by Thurlow [1925] in this system might be accounted for by this reaction. If such were the case the xanthine oxidase could no longer be regarded as supporting the Wieland view.

It therefore became important to ascertain whether the uptake of oxygen by the xanthine oxidase system was dependent upon the presence of such yellow pigments.

This question could be settled experimentally in the following ways. (1) By complete removal of yellow pigment from xanthine oxidase preparations. This should abolish the power of taking up oxygen. (2) By addition of yellow pigment to pigment-free preparations, which should increase the rate of oxygen uptake. (3) By determining the ratio, rate of methylene blue reduction/rate of oxygen uptake, in a number of xanthine oxidase preparations prepared in different ways,

and comparing its value with the pigment content of the preparations. The smaller the pigment content, the larger should be the ratio. (4) By determining the power of the oxidase to reduce the yellow pigment.

Experimental methods.

Preparations of xanthine oxidase.

A. *Caseinogen preparation* [Dixon and Thurlow, 1924, 1].

The enzyme is precipitated together with the caseinogen and fat by half-saturation of the milk with ammonium sulphate. The fat is removed from the dried precipitate by thorough extraction with ether.

B. *Cream preparation* [Toyama, 1933].

Cream is separated from milk. The fat is dissolved away with ether, and the protein residue is dried in the desiccator.

C 1. *Whey preparation* [Dixon and Kodama, 1926].

Fresh milk is clotted by rennin, the enzyme precipitated from the whey by half-saturation with ammonium sulphate and the precipitate extracted with ether and dried.

C 2. *Charcoal-treated whey preparation* [Dixon and Kodama, 1926].

A solution of the C 1 preparation is treated twice with Merck's medicinal charcoal, and the enzyme is precipitated from the clear filtrate with ammonium sulphate. Most of the protein and all the yellow pigment are removed by this procedure.

C 3. *Kaolin-treated whey preparation* [Dixon and Kodama, 1926].

The enzyme is adsorbed from a solution of the C 1 preparation at p_H 5 with kaolin and subsequently eluted from the kaolin with 1 % Na_2CO_3. It is then precipitated as before with ammonium sulphate. This procedure also removes most of the protein and all the yellow pigment.

C 4. *Fuller's earth-treated whey preparation.*

Fuller's earth is known to adsorb the yellow pigment strongly and is used for its concentration from milk. A solution of the C 1 preparation is treated twice with fuller's earth (Boots). The filtrate contains practically all the enzyme, no yellow pigment and about half the original protein. The enzyme is precipitated as before with ammonium sulphate.

D. *Sodium chloride preparation* (Dixon and Lemberg, unpublished).

The caseinogen and fat are precipitated from milk with sodium chloride, leaving the enzyme in solution, from which it is precipitated with ammonium sulphate. This gives a preparation of high activity containing a small amount of the yellow pigment. The complete details of the method will be published shortly.

Preparation of lactoflavine.

Ten litres of milk were acidified to p_H 4·6 with glacial acetic acid, and the caseinogen was filtered off. The filtrate was mixed with 500 g. of fuller's earth, which was filtered off after 1 hour and washed three times with 1 litre of distilled water and twice with 1 litre of ethyl alcohol. The lactoflavine was then eluted, following the procedure of Kuhn *et al.*, with a mixture of pyridine, acetic acid

and methyl alcohol. After centrifuging off the fuller's earth, the yellow solution was concentrated *in vacuo* to about 25 ml. and mixed with an equal volume of distilled water. The solution was then thoroughly extracted with ether, the yellow pigment remaining in the aqueous solution. The small precipitate which formed was filtered off. An equal volume of methyl alcohol was then added to the opalescent solution, causing the precipitation of a small amount of colloidal fuller's earth, which was filtered off. The yellow solution at this stage was perfectly clear and fluoresced strongly. It was then concentrated *in vacuo* to about 15 ml. and mixed with 10 volumes of acetone. The whole solution set to a gel, which could be easily broken up and filtered. The clear filtrate was again concentrated *in vacuo* to 15 ml., and phosphate solution (p_H about 7·4) was added until no more precipitate was formed. The precipitate was filtered off and the filtrate used in subsequent experiments. By colorimetric comparison with a lactoflavine solution of known concentration kindly given to us by Dr György it was found that the solution contained 50γ of lactoflavine per ml. The yield from 10 litres of milk was therefore 1 mg. of lactoflavine.

Detection of lactoflavine in xanthine oxidase preparations.

The dried enzyme preparations were extracted with small volumes of either methyl alcohol or pyridine, and the extracts were examined for greenish fluorescence in a strong beam of ultra-violet radiation, obtained from a mercury arc lamp with a nickel oxide filter. This test is exceedingly delicate, particularly when methyl alcohol is used, and 0·05γ of lactoflavine per g. of enzyme preparation can be detected with ease. In some cases aqueous solutions of the preparations were also tested for fluorescence.

Measurement of rate of reduction of methylene blue.

For the Thunberg technique to serve as a quantitative measure of the rate of reduction of methylene blue, the following conditions must be fulfilled. (1) The solutions must be completely freed from all traces of dissolved oxygen. (2) The substrate must not be mixed with the enzyme solution prior to evacuation of the tubes. (3) Temperature equilibrium must be attained by the tubes before the experiment begins.

The procedure adopted was as follows. Thunberg tubes of the Keilin type were used, and the hypoxanthine was placed in the hollow stopper. Four tubes were used for each determination. These were evacuated simultaneously with a water-pump, filled with oxygen-free nitrogen from a reservoir and allowed to come into equilibrium with the gas before being again exhausted. This process was twice repeated. The tubes were placed in a thermostat at 37°. The stoppers were worked in and the side-tubes filled with water in order to prevent air leakage. After three minutes the tubes were tilted simultaneously so as to mix the enzyme and substrate. By this procedure duplicates were found to agree to within 5 % in all cases.

The xanthine oxidase presents special difficulties in the determination of the rate of reduction of methylene blue, owing to the peculiar effects which are produced by changes of substrate concentration. Dixon and Thurlow [1924, 2] showed that over a certain range of hypoxanthine concentration the rate is maximum and independent of the concentration, but that with higher concentrations of substrate an inhibition ensues. A single determination, therefore, is insufficient to determine the true activity of the enzyme, and it is necessary in every case to work with two different concentrations of hypoxanthine, in order to ensure that the maximum rate is being measured.

The strength of the methylene blue solution used was determined by colorimetric comparison with a standard solution of a twice recrystallised specimen of pure medicinal methylene blue of known nitrogen and moisture content.

Measurement of rate of uptake of oxygen.

The measurements were carried out in the Barcroft apparatus at 37° by the usual technique [Dixon, 1934]. The hypoxanthine was placed in "Keilin tubes" and mixed with the enzyme only after temperature equilibrium was attained. Air, and not oxygen, was used in the flasks, as control experiments showed that the rate was independent of the partial pressure of oxygen within wide limits. Any traces of CO_2 were absorbed by KOH-papers in the usual way. Here again experiments with different hypoxanthine concentrations must be carried out, as the effects of substrate concentration are similar to those observed with methylene blue.

Procedure.

Suitable amounts of dried enzyme powder were dissolved in $M/5$ phosphate buffer (p_H 7·77) and diluted so as to give convenient rates of reduction. Barcroft and Thunberg experiments were carried out simultaneously, and each experiment was always done in duplicate.

Experimental results.

The yellow pigment can be removed from the oxidase preparations by adsorption with charcoal, kaolin or fuller's earth, and also by dialysis. For instance, one adsorption by any of these adsorbents is sufficient to remove the pigment from the C 1 preparation so completely that no trace can be detected by the fluorescence test when the oxidase is subsequently precipitated and 5 g. of the dried precipitate extracted with methyl alcohol. Nevertheless, after three successive treatments with charcoal the enzyme is found to have lost none of its power of taking up oxygen, and treatment with kaolin or fuller's earth likewise leaves the rate of oxygen uptake unaffected. It is therefore clear that the oxygen uptake does not depend upon the presence of lactoflavine.

In order to determine whether the pigment influences the rate of oxygen uptake in any way, the ratio, rate of methylene blue reduction/rate of oxygen uptake, was determined for the various preparations of the oxidase and compared with their content of yellow pigment. The results are summarised in Table I.

Table I.

Preparation	Degree of concentration of oxidase	Presence of lactoflavine	M.B. reduced in 20 mins. (mm.^{3} O_2)	O_2 absorbed in 20 mins. (mm.^{3})	Ratio
Milk	1	+ + + +	76	125	0·61
A	30	+ + +	19	34	0·56
B	400	−	36	63	0·57
C 1	500	+?	230	328	0·70
C 2	2000	−	98	174	0·56
C 3	2000	−	111	181	0·61
C 4	1000	−	87	119	0·73
D	2000	+	195	322	0·61

The figures given in the second column of the table represent very approximately the degree of concentration given by the different methods of preparation, *i.e.* they give the number of units of enzyme in 1 g. of the solid compared with 1 g. of the original milk. A *minus* sign in the third column means that no trace

of lactoflavine could be detected in 5 g. of the solid by the procedure given above. The amounts of methylene blue reduced have been expressed in mm.^{3} of oxygen, on the assumption that one molecule of methylene blue is equivalent to one atom of oxygen, in order to be able to compare the two rates. The figures given in the fourth column represent the amounts which 2·5 ml. of enzyme solution would reduce in 20 minutes, calculated from the observed rate of reduction of 1 ml. of methylene blue (M/1500). The amounts of oxygen given in the fifth column are obtained from the initial linear portions of the oxygen uptake curves and represent the amounts absorbed by 2·5 ml. of the enzyme solutions in 20 minutes at the same temperature. Since the solutions of the various preparations of enzyme were arbitrarily diluted to different extents, so as to give convenient rates, the figures given in columns 4 and 5 give no index of the relative activities of the different preparations in the solid state and cannot be compared with one another.

It will be seen that the ratio of the two velocities remains remarkably constant, in spite of wide variations in the content of lactoflavine.

The addition of concentrated lactoflavine solution to preparations which had been freed from yellow pigment by dialysis or adsorption produced no increase in the rate of oxygen uptake, as shown in Tables II and III.

Table II.

Barcroft manometer	1	2	3	4
Dialysed oxidase (preparation A) (ml.)	2·5	2·5	2·5	2·5
Hypoxanthine (5 mg./ml.) (ml.)	0·2	0·2	0·2	0·2
Lactoflavine (0·00016 M) (ml.)	—	—	0·2	0·2
Water (ml.)	0·3	0·3	0·1	0·1
O_2 uptake in 20 mins. (mm.^{3})	38·0	37·4	35·7	36·6

Table III.

Barcroft manometer	1	2	3	4
Oxidase (preparation C 2) (ml.)	2·5	2·5	2·5	2·5
Hypoxanthine (5 mg./ml.) (ml.)	0·2	0·2	0·2	0·2
Lactoflavine (0·00016 M) (ml.)	—	—	0·2	0·2
Water (ml.)	0·3	0·3	0·1	0·1
O_2 uptake in 10 mins. (mm.^{3})	81·7	74·5	79·6	79·8

Table IV.

Thunberg tube	1	2	3	4
Oxidase (preparation A) (ml.)	1·0	1·0	1·0	1·0
Hypoxanthine (5 mg./ml.) (ml.)	0·2	0·2	0·2	0·2
Lactoflavine (0·00016 M) (ml.)	1·0	1·0	—	—
Methylene blue (0·00034 M) (ml.)	—	—	1·0	1·0
Reduction time	35 mins.	36 mins.	70 secs.	70 secs.

The power of the xanthine oxidase to reduce lactoflavine anaerobically was then tested by comparing the times of reduction of 60γ added lactoflavine and methylene blue in Thunberg tubes. The times given in Table IV are those for complete reduction as shown by complete disappearance of colour. It can be easily calculated that lactoflavine is reduced with a velocity of only 1/64th of that of methylene blue reduction.

Discussion.

The results given above show clearly that lactoflavine plays no part in the oxygen uptake by the xanthine oxidase. The rate of uptake is unaffected alike by complete removal and by addition of the pigment, and the ratio of the reaction rates with methylene blue and oxygen does not change with the pigment content.

An incredibly high catalytic activity would have to be assumed if traces of the yellow pigment, too small to be detected, are to account for the observed oxygen uptake. In preparation C 3, for example, certainly less than $0{\cdot}05\gamma$ per g. was present. 10 mg. of the preparation, containing less than $0{\cdot}0005\gamma$ of lactoflavine, were used in the experiment given in Table I, and took up 181 mm.^{3} in 20 minutes, or 9 mm.^{3} per minute. As $0{\cdot}0005\gamma$ of lactoflavine is equivalent to 0·000015 mm.^{3} of oxygen, the lactoflavine would have to be reduced and oxidised 600,000 times per minute in order to account for the oxygen uptake, whereas Warburg and Christian found that the corresponding catalytic flavines of bottom yeast and lactic acid bacteria were reduced and oxidised only 24 and 30 times per minute respectively.

The observed rate of reduction of lactoflavine by xanthine oxidase also shows that it cannot account for the oxygen uptake. Table IV shows that an amount of lactoflavine corresponding to 1·8 mm.^{3} of oxygen was reduced in 35 minutes. In the same period this solution of oxidase would have taken up about 210 mm.^{3} of oxygen, so that it is clear that even this relatively large amount of pigment could only account for less than 1 % of the oxygen uptake.

Since Dixon and Thurlow showed that the xanthine oxidase differed from the majority of the oxidases, in that its ability to react with molecular oxygen was not inhibited by HCN, it has been regarded, in common with the other cyanide-stable respiration systems, as a case in which the oxygen reacted directly with the activated substrate molecules in accordance with Wieland's theory. Warburg and Christian [1933] have, however, shown that at any rate in some of these cases, which the haematin catalysis theory could not explain, the oxygen uptake could be accounted for by the action of catalytic flavines (which are resistant to cyanide), the oxygen reacting with these and not directly with the organic substrates.

Warburg and Christian assert that, just as the Wieland theory is wrong in all those cases in which the respiration is inhibited by HCN (for the oxygen then reacts, not with the substrate, but with the reduced form of a haematin), it is no less wrong in those (rarer) cases in which the respiration is not inhibited by HCN (for the oxygen then reacts, again not with the substrate, but with the leuco-form of the flavine); and they state that the idea of a direct reaction of molecular oxygen with the substrate must now be abandoned.

In the xanthine oxidase, however, we have an oxidase system whose oxygen uptake is due neither to haematin nor to flavine, but apparently to a direct reaction of oxygen with the activated substrate molecules.

Summary.

The ability of the xanthine oxidase of milk to react with molecular oxygen is not dependent upon the presence of lactoflavine. In this system the molecular oxygen probably reacts directly with the activated substrate molecules.

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REFERENCES.

Dixon (1934). Manometric methods. (Cambridge.)
—— and Kodama (1926). *Biochem. J.* **20**, 1104.
—— and Thurlow (1924, 1). *Biochem. J.* **18**, 971.
—— —— (1924, 2). *Biochem. J.* **18**, 976.
—— —— (1925). *Biochem. J.* **19**, 672.
Kuhn, György and Wagner-Jauregg (1933). *Ber. deutsch. chem. Ges.* **66**, 1034.
Stern (1933). *Nature*, **132**, 784.
Thunberg (1917). *Skand. Arch. Physiol.* **35**, 163.
Thurlow (1925). *Biochem. J.* **19**, 175.
Toyama (1933). *J. Biochem.* (Japan), **17**, 131.
Warburg and Christian (1932). *Biochem. Z.* **254**, 438.
—— —— (1933). *Biochem. Z.* **266**, 377.

XXXIII. STUDIES IN TISSUE METABOLISM.
III. THE EFFECT OF OXIDISED 1:2:5:6-DIBENZANTHRACENE.

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By means of the Thunberg technique oxidation products of carcinogenic hydrocarbons were shown to inhibit lactic and succinic dehydrogenases of yeast and muscle extracts in the presence of methylene blue as hydrogen acceptor [Boyland, 1933]. Qualitative examination of the effect on the oxidation of *p*-phenylenediamine by washed tissue indicated that these hydrocarbons did not inhibit indophenol oxidase. The enzymes used in the previous experiments had been used in the form of extracts. The object of the present work was the investigation of the effect of a water-soluble oxidation product of the carcinogenic hydrocarbon, 1:2:5:6-dibenzanthracene [Cook *et al.*, 1932] on the respiration and anaerobic glycolysis of surviving animal tissues (normal organs and tumours of rat and mouse) using the Warburg technique. The tissues were suspended in Ringer-phosphate or $M/15$ phosphate solutions (p_H 7·3), either in slices less than 0·4 mm. thick or finely minced, in the vessels of a Warburg apparatus; the amounts of oxygen absorbed and the anaerobic glycolysis were measured in the presence of varying concentrations of the oxidation product.

Experimental.

1:2:5:6-Dibenzanthracene (0·1 % in benzene) was oxidised by passing a stream of oxygen through the solution while exposed to ultra-violet light for 48 hours. A light brown precipitate was formed, and the solution became darker. The precipitate and solution were extracted with aqueous $2N$ NaOH, and the extract was precipitated with cold hydrochloric acid. The brown precipitate was washed with water and dried; weighed quantities were dissolved either (1) in phosphate buffer solution ($M/5$; final p_H 7·3) for determination of respiration or (2) in 0·82 % Na_2CO_3 solution, neutralised with CO_2 gas, for measurement of glycolysis. In some cases the precipitate never entirely dissolved; this was due to the instability of the oxidation product, which in certain circumstances partially changes to an insoluble substance. Concentrations quoted below are therefore only approximate.

Measurement of metabolism.

The standard Warburg apparatus was used, with simple conical vessels with inset trough and side-arm. For respiration, 0·1 ml. of $2N$ NaOH was placed in the central trough, and the tissue in $M/15$ phosphate or Ringer-phosphate was contained in the main vessel. In some cases the oxidised dibenzanthracene solutions and substrate were also placed in the main vessel. When tissue slices were used it was however found advisable to measure the respiration in Ringer

alone for a certain time ($\frac{1}{2}$ to 1 hour) before adding the substrates and different concentrations of oxidation product from the side vessels. The reason for this was that variations up to 15 % occurred in the respiration of tissues from any one animal. Brain, and tumours containing much blood or necrotic material, showed variations outside these limits. The respiration was in all cases measured in oxygen which was passed through the vessels for 20 seconds before closing the taps. Tissues were minced finely with scissors and 0·1 or 0·2 g. suspended in $M/15$ phosphate in the Warburg vessels, or slices were prepared according to Warburg [1923] and the dry weights determined at the end of the experiment. In each set of experiments there was one thermobarometer, one vessel containing a sample of tissue in Ringer-phosphate (or phosphate) alone and another the same with lithium lactate or sodium succinate (final concentration $M/100$). The remaining vessels contained in addition varying concentrations of oxidised dibenzanthracene.

A typical experiment was as follows:

		Side vessel			
Vessel No.	Main vessel Ringer-phosphate ml.	Lactate ml.	Oxidised dibenzanthracene in phosphate ml.	Phosphate ml.	Inset 2N NaOH ml.
1	1	—	—	—	—
2	0·6	—	—	0·3 +0·1 H_2O	0·1
3	0·6	0·1	—	0·3	0·1
4	0·6	0·1	0·1	0·2	0·1
5	0·6	0·1	0·2	0·1	0·1
6	0·6	0·1	0·3	—	0·1

The vessels after being filled with oxygen were shaken for 15 minutes in the thermostat to reach temperature equilibrium, and then readings were taken every 10 or 15 minutes, generally for an hour, before tipping in the solutions from the side vessels. When minced tissue was used it was found unnecessary to determine the normal respiration as there were negligible differences between duplicate experiments; in these cases all the solutions were from the beginning in the main vessels with the tissue. For determination of glycolysis, the tissue was suspended in Ringer-bicarbonate containing glucose, and the dibenzanthracene derivative was dissolved in bicarbonate. A mixture of 95 % nitrogen and 5 % carbon dioxide was passed through the vessels for a minute at the beginning of an experiment.

A few sets of experiments on respiration and glycolysis were carried out under aseptic conditions, and cultures were made at the end of the experiment to test the sterility. In no case did the comparison of these and other experiments show differences which could be ascribed to bacterial contamination.

Results.

A. *Normal tissues.*

Muscle. Minced voluntary muscle of the rat in $M/15$ phosphate was used for the determination of respiration and anaerobic glycolysis at 25°. In one series of experiments on respiration, diaphragm muscle (rat) at 37·5° in Ringer-phosphate was used.

The tables show that a concentration of 0·02 % of oxidised dibenzanthracene reduced the oxygen consumption by a third in the first hour, and 0·3 % by 55 %.

Table I. *Minced rat muscle in M/15 phosphate* (p_H *7·3*) *at 25°.*

	Q_{O_2} Concentration of oxidised dibenzanthracene			
Hour	0	0·01 %	0·02 %	0·08 %
1	37·5	31·0	25·0	20·2
2	18·7	17·5	11·2	7·2
3	7·0	7·5	4·0	4·0

Table II. *Minced rat muscle in Ringer at 25°.*

	Q_{O_2} in Ringer-phosphate Concentration of oxidised dibenzanthracene		
Hour	0	0·1 %	0·3 %
1	25·0	14·3	11·3
2	20·5	9·5	4·0
3	12·8	7·5	2·0

	$Q_M^{N_2}$ in Ringer-bicarbonate Concentration of oxidised dibenzanthracene			
Hour	0	0·1 %	0·2 %	0·3 %
1	21·3	18·8	20·0	4·6
2	7·5	6·3	3·8	0·0

Table III. *Rat diaphragm in Ringer-phosphate at 37·5°.*

Hour	Q_{O_2}			
1	11·6	11·5	12·5	10·0
	Concentration of oxidised dibenzanthracene			
	0	0·03 %	0·06 %	0·09 %
2	9·7	9·0	7·7	5·7
3	8·5	8·6	5·8	5·2
4	7·9	7·5	5·4	3·8
	% decrease calculated on 1st hour			
2	16	22	38	43
3	27	25	54	48
4	33	35	57	62

In 3 hours this concentration almost stopped respiration and had completely arrested glycolysis within 2 hours.

As the glycolysis of minced rat muscle was inhibited by the oxidised 1:2:5:6-dibenzanthracene, a few experiments on the effect of the substance on glycolysis of rat muscle extract were carried out, with the co-operation of Dr C. A. Mawson. The muscle extract was made according to the method used by Meyerhof [1930]; although Meyerhof states that rat muscle yields an inactive extract Mawson (unpublished results) has been able to obtain glycolytically active rat muscle extracts. The results of experiments on undialysed and dialysed extracts showing pronounced inhibition of the lactic acid formation are given in Tables IV (*a*) and (*b*).

Table IV (*a*). *5 ml. undialysed rat muscle extract incubated 2 hours at 37°.*

			Oxidised 1:2:5:6-dibenz-anthracene %	mg. lactic acid	% inhibition of lactic acid formation
Unincubated				9·76	
Incubated with adenylpyrophosphate + $MgCl_2$				10·13	
,,	,,	+glycogen		17·64	
,,	,,	,,	+0·002	17·37	3·6
,,	,,	,,	+0·004	16·05	21
,,	,,	,,	+0·012	11·85	77

Table IV (*b*). *5 ml. rat muscle extract dialysed for 3 hours at 0°, incubated 2 hours at 37°.*

			Oxidised 1:2:5:6-dibenz-anthracene %	mg. lactic acid	% inhibition of lactic acid formation
Unincubated extract				1·34	
Incubated with adenylpyrophosphate and $MgCl_2$				1·66	
,,	,,	+glycogen		5·12	
,,	,,	,,	+0·002	4·39	21
,,	,,	,,	+0·004	3·27	54
,,	,,	,,	+0·012	2·37	78

Liver. All samples in the following tables, except those marked "No lactate" (or "No succinate") had an addition of $M/100$ lactate (or $M/100$ succinate) after an hour.

Table V. *Rat liver slices in Ringer-phosphate at 37°.*

	Q_{O_2}				
		Concentration of oxidised dibenzanthracene			
Hour	No lactate 0	0	0·002 %	0·005 %	0·01 %
1	4·3	7·3	7·5	6·2	3·4
2	3·4	6·6	6·5	5·0	2·0
3	3·3	6·2	5·0	4·7	1·6

Table VI. *Rat liver slices in Ringer-phosphate at 37°.*

Hour	Q_{O_2}				
1	9·3	9·1	8·2	9·3	8·3
		Concentration of oxidised dibenzanthracene			
	No lactate 0	0	0·1 %	0·2 %	0·3 %
2	9·3	10·0	8·6	9·8	7·1
3	8·5	8·7	8·1	8·9	6·2
4	7·8	8·1	7·5	7·6	5·7
	% difference, calculated on 1st hour				
2	0	+10	+5	+5	−14
3	−8	−4	−1	−4	−25
4	−16	−11	−8	−18	−31

Tables V and VI are typical of ten experiments on liver. Table V shows that the respiration was increased 70 % by the addition of lactate, and that the dibenzanthracene derivative greatly reduced the oxygen uptake. In the experiment represented by Table VI, on the other hand, the respiration was very slightly increased by lactate, and the inhibition was also less. The mean of six sets of experiments shows almost equal increase of respiration due to added lactate, and decrease due to the presence of oxidised dibenzanthracene, indicating that it is mainly lactic oxidation which is inhibited.

Kidney. Sections from rat kidney cortex were used to investigate the effect of the oxidised hydrocarbon on lactate and on succinate oxidation. As the normal respiration of kidney is higher than that of liver thinner sections (0·15–0·3 mm.) were used.

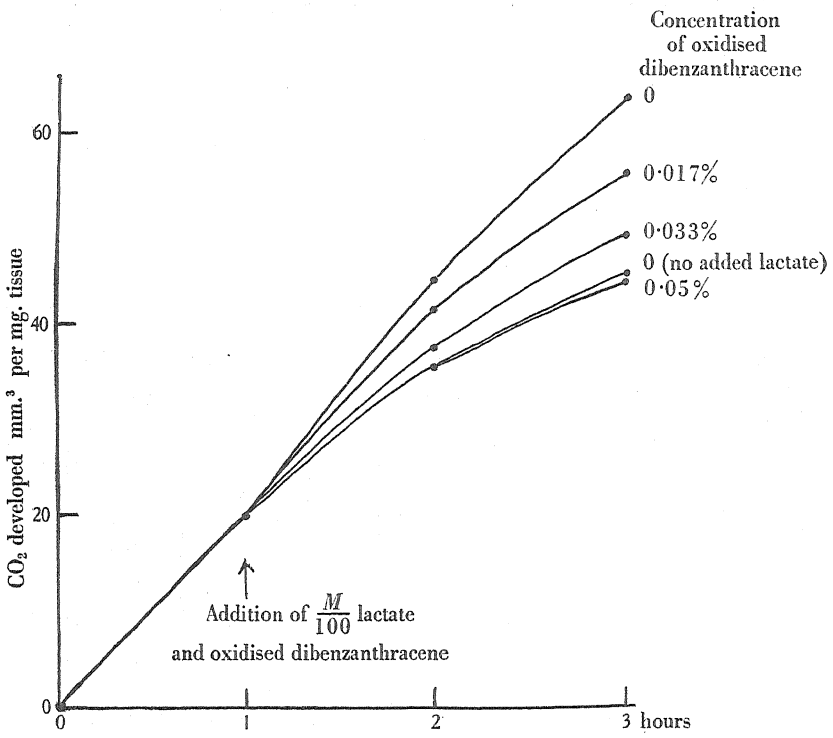

Fig. 1. Respiration of kidney slices in Ringer-phosphate at 37·5°.

Table VII. *Rat kidney slices in Ringer-phosphate (with lactate) at 37·5°.*

Hour	Q_{O_2}								
1	20·7	19·0	21·5	22·5	20·0	22·5	22·6	25·5	21·9
		Concentration of oxidised dibenzanthracene							
	No lactate 0	0	0	0·005 %	0·01 %	0·02 %	0·05 %	0·1 %	0·2 %
2	16·2	26·5	25·5	28·0	23·5	23·0	22·2	25·0	17·8
3	8·3	17·2	20·0	20·2	16·5	16·8	15·0	17·5	12·2
	% difference calculated on 1st hour								
2	−22	+40	+19	+22	+17	+2	−2	−2	−19
3	−60	−9	−7	−11	−17	−25	−34	−31	−45

Table VIII. *Rat kidney slices in Ringer-phosphate (with succinate) at 37·5°.*

Hour	Q_{O_2}				
1	16·8	18·0	17·8	15·5	16·8
		Concentration of oxidised dibenzanthracene			
	No succinate	0	0·017 %	0·033 %	0·05 %
2	13·9	31·5	25·1	18·8	21·8
3	8·8	16·5	15·0	11·2	11·8
	% difference, calculated on 1st hour				
2	−17	+75	+41	+21	+30
3	−48	−8	−16	−28	−30

Tables VII and VIII show that a concentration of 0·02 % of the benzanthracene derivative has a measurable effect on lactate oxidation. Table VII shows that within the experimental error increasing concentrations from 0·001 up to 0·2 % show a steadily increasing effect. A concentration of 0·033 % has an inhibitory effect on succinic oxidation.

Brain. A modification of the technique used by Quastel [1932] was employed in a few sets of experiments on minced rat's brain. The tissue was allowed to respire in phosphate solution for 2 hours before the lactate and the solutions of oxidised dibenzanthracene were added. The respiration fell rapidly during the first hour and then became more uniform, though still falling slowly. The normal respira-

Table IX. *Rat brain minced in M/15 phosphate (dry weights taken as 1/5 wet weights) at 37·5°.*

Hour	Q_{O_2}				
2	19·2	17·7	17·0	18·0	17·5
		Concentration of oxidised dibenzanthracene			
	No lactate	0	0·005 %	0·01 %	0·02 %
3	11·1	18·5	21·2	17·9	16·5
4	8·1	16·8	19·8	17·2	11·8
	% difference, calculated on 2nd hour				
3	−42	+4	+25	−1	−5
4	−58	−5	+16	−4	−33

Table X. *Rat brain slices in Ringer-phosphate (from dry weights, brain dried at 50°) at 37·5°.*

Hour	Q_{O_2}				
1	6·0	5·0	6·5	7·8	5·1
		Concentration of oxidised dibenzanthracene			
	No lactate	0	0·017 %	0·033 %	0·05 %
2	3·3	5·4	6·3	8·0	4·2
3	1·4	4·3	5·0	5·8	2·6
	% difference, calculated on 1st hour				
2	−45	+8	−3	+2	−18
3	−77	−14	−23	−25	−49

Table XI. *Rat brain slices in Ringer-bicarbonate (from dry weights, brain dried at room temperature) at 37·5°.*

	$Q_M^{N_2}$ Concentration of oxidised dibenzanthracene			
Hour	0	0·03 %	0·1 %	0·3 %
1	5·7	6·4	4·4	3·9
2	3·6	3·9	2·3	1·5

Table XII. *Rat brain slices in Ringer-bicarbonate (from dry weights, brain dried at room temperature) at 37·5°.*

	$Q_M^{N_2}$ Concentration of oxidised dibenzanthracene			
Hour	0	0·03 %	0·1 %	0·3 %
1	6·6	6·0	6·2	5·4
2	5·6	4·3	5·0	3·5

tion was taken as that during the second hour and the respiration in subsequent hours compared with it. Respiration and glycolysis were also investigated for brain slices in Ringer. As the glycolytic activity of brain decreased rapidly with time, the oxidised dibenzanthracene was in contact with the tissue from the beginning in the glycolysis experiments. Though this lessens the accuracy of the determinations the effect found was so large as to be outside the possible error. The sections were dried either at 50° or at room temperature in a desiccator. Tables IX–XII show that the minimum concentration to show an effect is about 0·02 %.

B. *Malignant tissues.*

Jensen's rat sarcoma (J.R.S.), and C 63[1], a transplantable mammary carcinoma of the mouse, were used in this work. In addition three strains of transplantable tumours produced by pure hydrocarbons were studied, and their respiration and anaerobic glycolysis measured for the first time. These latter tumours which are spindle-celled sarcomas were produced here by subcutaneous injection of solutions in lard of 1:2:5:6-dibenzanthracene (tumour R 10, rat) or of 5:6-*cyclo*penteno-1:2-benzanthracene (tumour M 154, mouse), or of 1:2:5:6-dibenzanthracene in lard and milk (tumour M 4, mouse). The first of these has now reached the 51st, the second the 44th and the third the 83rd grafted generation.

Jensen's rat sarcoma. In addition to experiments similar to those described for other tissues, in a few cases considerable precautions were taken to avoid bacterial infection. The solutions used were all sterilised and measured out from sterile pipettes, the Warburg vessels and Petri dishes were autoclaved and the tumours removed aseptically from the rats. Sterile finger-stalls were used when cutting the sections. The gases were passed through cotton wool plugs, and the parts of the manometer through which they passed were rinsed out with absolute alcohol. Cultures were taken from the liquids at the end of the experiments and examinations made after 18 hours' incubation. Table XIV shows the result of one such experiment, with the bacteriological report.

[1] We are indebted to the Imperial Cancer Research Fund for this tumour.

Table XIII. *J.R.S. slices at 37·5°.*

Hour	Concentration of oxidised dibenzanthracene: 0	0·1 %	0·2 %	0·3 %
	Q_{O_2} in Ringer-phosphate			
1	12·2	8·6	9·8	8·4
2	8·8	8·6	9·0	7·3
3	7·0	7·4	6·6	5·0
	$Q_M^{N_2}$ in Ringer-bicarbonate			
1	26·2	18·5	15·1	21·0
2	19·0	17·4	11·7	11·4
3	14·4	12·8	5·3	5·8

Table XIV. *J.R.S. slices at 37·5°, aseptic technique.*

(Roman numerals refer to bacteriological report.)

Hour	Q_{O_2} in Ringer-phosphate: No lactate I	Concentration of oxidised dibenzanthracene: II 0	III 0·1 %	IV 0·2 %	V 0·3 %
1	10·0	10·4	8·2	7·4	7·2
2	7·8	8·7	7·3	6·3	4·5
3	7·1	6·9	6·5	5·4	3·1

	$Q_M^{N_2}$ in Ringer-bicarbonate; Concentration of oxidised dibenzanthracene: VI 0	VII 0·1 %	VIII 0·2 %	IX 0·3 %
1	19·2	14·0	15·0	9·5
2	14·0	11·1	11·4	6·1
3	8·0	5·7	4·6	4·1

Sterility test on fluid from experiment shown in Table XIV after 18 hours' incubation.

Vessel I. Slight growth of streptococci and *B. subtilis*.
Vessel II. Moderate growth of staphylococci and streptococci.
Vessel III. Very slight growth of staphylococci.
Vessel IV. Moderate growth of streptococci and staphylococci.
Vessel V. Very slight growth of staphylococci.
Vessel VI. Very slight growth of *B. subtilis*.
Vessel VII. Sterile.
Vessel VIII. Sterile.
Vessel IX. Sterile.

Eighteen hours' incubation after the conclusion of the experiment constitutes a somewhat severe test of aseptic technique. It is evident in Table XIV that such degrees of contamination as had occurred show no parallelism with the chemical changes measured (*e.g.* vessels III, IV, V).

Dickens [1931] quotes results for J.R.S.:

Warburg, $Q_{O_2}=9$, $Q_M^{N_2}=34$;
Crabtree, $Q_{O_2}=8$, 11, $Q_M^{N_2}=32$.

Rat sarcoma 10. Tables XV and XVI, typical of experiments on this tumour, show that concentrations of less than 0·1 % oxidised dibenzanthracene produce

no inhibition of oxygen uptake, within the times of the experiments (2–3 hours) and that higher concentrations (0·2, 0·3 %) show an effect only after one hour.

Table XV. *R. 10 slices in Ringer-phosphate at 37·5°.*

Hour	Q_{O_2}				
1	12·5	12·2	12·6	13·0	13·2
		Concentration of oxidised dibenzanthracene			
	No lactate	0	0·017 %	0·033 %	0·05 %
2	12·2	12·2	12·6	11·5	13·5
3	11·1	11·7	10·7	10·7	12·5
	% difference, calculated on 1st hour				
2	−2	0	0	−12	+2
3	−11	−4	−15	−18	−5

Table XVI. *R. 10 slices in Ringer-phosphate at 37·5°.*

Hour	Q_{O_2}			
1	13·8	15·0	12·7	13·8
	Concentration of oxidised dibenzanthracene			
	0	0·1 %	0·2 %	0·3 %
2	12·4	13·4	11·4	11·4
3	10·4	7·9	4·8	3·5
4	10·0	6·7	2·7	2·1
	% difference, calculated on 1st hour			
2	−11	−11	−10	−17
3	−25	−47	−62	−75
4	−28	−55	−80	−85

Table XVII. *R. 10 slices in Ringer-bicarbonate at 37·5°.*

Hour	$Q_M^{N_2}$			
1	19·0	18·3	22·3	22·0
	Concentration of oxidised dibenzanthracene			
	0	0·1 %	0·2 %	0·3 %
2	14·5	13·8	12·0	10·0
3	11·6	11·7	9·2	5·7
4	6·2	7·1	6·2	4·2
	% difference, calculated on 1st hour			
2	−23	−25	−45	−55
3	−39	−35	−57	−74
4	−67	−61	−73	−82

Mouse sarcoma M 154. It was difficult to get sections of this tumour completely free from necrotic material, and as the method of calculation used makes use of the absolute value of the initial respiration of each sample, no great trouble was taken to ensure the absence of necrosis from the slices used. This accounts for the considerable differences in amount of respiration found. It will be seen that in the glycolysis experiments the differences in the first hour (after adding the oxidised dibenzanthracene) are greater than could reasonably be accounted for by the presence of some necrotic material.

Table XVIII. *M 154 slices in Ringer-phosphate at 37·5°.*

Hour	Q_{O_2}				
1	10·0	9·8	9·5	8·4	8·0
		Concentration of oxidised dibenzanthracene			
	No lactate	0	0·017 %	0·033 %	0·05 %
2	7·3	9·6	7·8	8·0	7·4
3	5·8	7·5	6·5	6·3	5·0
4	3·7	6·7	5·4	5·4	4·3
5	3·1	5·8	5·1	4·7	2·6
	% difference, calculated on 1st hour				
2	−27	−2	−18	−5	−8
3	−42	−23	−31	−25	−38
4	−63	−32	−43	−35	−47
5	−69	−41	−46	−44	−68

Table XIX. *M 154 slices at 37·5°.*

Hour	Q_{O_2} in Ringer-phosphate			
1	11·5	12·1	8·6	9·3
	Concentration of oxidised dibenzanthracene			
	0	0·1 %	0·2 %	0·3 %
2	11·0	9·7	5·2	1·8
3	9·1	8·4	2·8	1·5
	% difference, calculated on 1st hour			
2	−4	−20	−40	−81
3	−21	−30	−68	−84
	$Q_{M}^{N_2}$ in Ringer-bicarbonate			
1	21·5	12·8	10·6	7·8
2	18·4	12·2	8·3	6·2
3	17·4	10·3	6·7	5·7

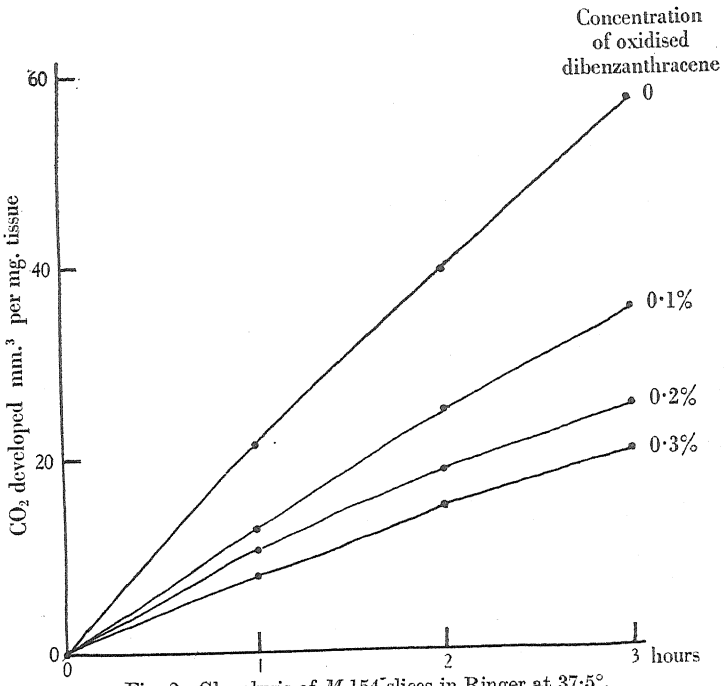

Fig. 2. Glycolysis of *M* 154 slices in Ringer at 37·5°.

Mouse sarcoma M 4. Table XX shows one of three similar experiments on M 4. A concentration of 0·033 % produced an inhibitory effect within 3 hours and 0·05 % after one hour. It is thus rather more susceptible than R 10 and J.R.S.

Table XX. *M 4 slices in Ringer-phosphate at 37·5°.*

Hour	Q_{O_2}				
1	9·0	10·6	9·5	9·0	10·5
		Concentration of oxidised dibenzanthracene			
	No lactate	0	0·017 %	0·033 %	0·05 %
2	8·4	9·9	8·9	9·0	9·7
3	7·1	8·5	7·9	7·5	7·0
4	6·2	7·2	6·1	5·0	3·8
	% difference, calculated on 1st hour				
2	−7	−6	−6	0	−8
3	−20	−20	−17	−17	−33
4	−31	−32	−36	−45	−64

Mouse carcinoma C 63. This tumour is vascular and has a small growing edge of sound tissue; sections were therefore difficult to cut free from necrotic material. This tumour appears to be rather more susceptible to the action of the oxidised dibenzanthracene than the others studied, as inhibition of respiration is observable after one hour even with a 0·033 % solution and 0·1 % produces almost the maximum inhibition (Tables XXI–XXIII).

Table XXI. *C 63 slices in Ringer-phosphate at 37·5°.*

Hour	Q_{O_2}				
1	8·0	11·5	8·3	10·6	12·5
		Concentration of oxidised dibenzanthracene			
	No lactate	0	0·017 %	0·033 %	0·05 %
2	6·7	10·5	7·2	7·2	8·0
3	5·4	8·2	6·6	6·4	6·7
4	3·7	6·8	4·7	4·5	4·8
	% difference, calculated on 1st hour				
2	−16	−9	−13	−32	−36
3	−32	−29	−20	−40	−46
4	−54	−41	−43	−58	−62

Table XXII. *C 63 slices in Ringer-phosphate at 37·5°.*

Hour	Q_{O_2}				
1	18·0	12·8	15·2	11·2	17·6
		Concentration of oxidised dibenzanthracene			
	No lactate	0	0·1 %	0·2 %	0·3 %
2	10·2	8·6	8·0	5·3	7·5
3	7·4	5·5	4·8	2·8	3·8
	% difference, calculated on 1st hour				
2	−43	−33	−47	−53	−56
3	−59	−57	−70	−74	−78

Table XXIII. *C 63 slices in Ringer-bicarbonate at 37·5°.*

Hour	$Q_M^{N_2}$			
1	15·5	19·4	12·2	14·3
	Concentration of oxidised dibenzanthracene			
	0	0·1 %	0·2 %	0·3 %
2	18·2	16·1	8·4	8·5
3	15·7	10·2	6·4	5·2
	% difference, calculated on 1st hour			
2	+17	−17	−31	−41
3	+1	−47	−48	−63

Conclusion.

The tables given above show that oxidised 1:2:5:6-dibenzanthracene acts as an inhibitor of oxidation and glycolysis in all the tissues studied. There is no remarkable difference between the effects on normal and on malignant tissues, except that the malignant tissues with the exception of C 63 are more slowly inhibited. A concentration of 0·2 % in every tissue examined produces a measurable diminution of metabolism; in the case of kidney and muscle 0·02 % is effective. Boyland [1933], using the methylene blue technique, found that a 0·002 % solution of the hydrocarbon produced after oxidation more than 50 % inhibition of lactic dehydrogenase from yeast, but the quantity of this enzyme present was probably much smaller than in the present experiments.

As all known reactions involving enzymes take place in aqueous solution, it seems probable that carcinogenic hydrocarbons are altered in the body to form water-soluble compounds before they can produce biological effects. The oxidation products of these hydrocarbons produced by oxygen in ultra-violet light are water-soluble and have a large inhibitory effect on oxidation processes. The present work shows that the action of the oxidised 1:2:5:6-dibenzanthracene on malignant cells is very similar to that on normal tissues. It is possible that the production of tumours by these carcinogenic compounds involves an oxidation of the compound, either to a product identical with that isolated and used in the foregoing experiments, or to a less stable intermediate oxidation product. Subcutaneous injections of 0·4 % solutions of the oxidation products used in the present work have not yet, after 14 weeks, produced any tumours in rats or mice.

Summary.

The effect of a water-soluble oxidation product of 1:2:5:6-dibenzanthracene on the metabolism has been studied in respect of the following rat tissues: 1. Muscle, respiration and glycolysis: 2. Liver, respiration: 3. Kidney, respiration: 4. Brain, respiration and glycolysis: 5. Jensen's sarcoma and rat sarcoma 10, respiration and glycolysis; and of the following mouse tissues: 6. Sarcoma M 154, respiration and glycolysis: 7. Sarcoma M 4, respiration: 8. Carcinoma 63, respiration and glycolysis.

In all cases an inhibitory effect was found.

Our thanks are due to Dr C. C. Bryson, Assistant Pathologist to the Cancer Hospital, for his bacteriological investigations on the samples submitted to him; and also to Prof. Kennaway for his interest and encouragement during the course of the work here described.

REFERENCES.

Boyland (1933). *Biochem. J.* **27**, 791.
Cook, Hieger, Kennaway and Mayneord (1932). *Proc. Roy. Soc. Lond.* B **111**, 455.
Dickens (1931). *Cancer Rev.* **6**, 57.
Meyerhof (1930). Die Chemischen Vorgänge im Muskel. (Berlin, J. Springer.)
Quastel (1932). *Biochem. J.* **26**, 725.
Warburg (1923). *Biochem. Z.* **142**, 317.

XXXIV. THE METABOLISM OF GALACTOSE.
II. THE BEHAVIOUR OF THE RAT TOWARDS MODERATE AMOUNTS OF GALACTOSE.

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The immediate purpose of the work reported in this and the previous paper [Harding and Grant, 1932–33] has been to trace as far as possible, by methods of direct analysis, the fate of orally administered galactose. The use of galactose-acclimatised yeast [Harding and Grant, 1931–32] or *S. marxianus* [Harding, Nicholson and Grant, 1932–33] as an analytical reagent enables free galactose in extracts or preparations of blood, urine and tissues to be estimated with precision. The previous paper showed that the oral administration of moderate amounts of galactose (40 g.) to adult man resulted in a rapid entrance of galactose into the blood stream, with a peak amount at 30–60 mins. followed by a fall to zero by the end of 2 hours. A small increase in arterial blood-glucose was also noted. The urine contained amounts not greater than 2 g. galactose. With this amount of galactose, all the sugar was disposed of in 2 hours, but with a larger dose (80 g.) the excretion into the urine sometimes continued until the fourth hour. No evidence was found in the blood or urine of any new reducing compound formed from galactose, nor did the urine show any trace of non-reducing substance which yielded galactose on hydrolysis, beyond those small, and as yet doubtful, amounts demonstrated in the fasting condition by Harding and Grant [1931–32]. The present communication traces the absorption of galactose from the intestine of the rat and its disappearance from the tissues.

Experimental and analytical methods.

Young adult white rats (both sexes) were starved for 24 hours. To Series A was given by stomach tube [Cori, 1925] 1 ml. of 10 % galactose solution. The rats were placed in a cage with provision for urine collection. Five minutes previous to the desired time 15–20 mg. amytal were injected. At the end of 5, 20, 60 or 180 mins. from the administration of the galactose, the alimentary tract was ligatured at the oesophagus and anus and removed. Any urine was removed from the bladder by a small syringe, the bladder being washed out 3 or 4 times with distilled water. The muscle specimen, if desired, was taken from the hind-leg by dissecting away the skin, freezing by solid CO_2 *in situ* and then removing. The liver, if desired, was removed and placed at once in solid CO_2. If the whole animal was desired, it was frozen by solid CO_2 immediately after the removal of the urine. In a few instances a blood specimen was taken from the tail previous

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to the freezing. To Series B was given 1 ml. of 20 % galactose solution, and the animals were examined at the end of 20, 60, 120 and 180 mins. A few control animals were examined at the end of 20 mins.

Preparation previous to analysis. The excised alimentary tract was at once opened throughout its entire length and washed out with distilled water three or four times, gently macerating the interior lining. The extract was treated with 10 % sodium tungstate and 2/3 N H_2SO_4. After standing for a few minutes this was filtered. The filtrate was shaken with purified Lloyd's reagent [Hamilton, 1928] and refiltered. The frozen muscles were weighed, dropped into 15 ml. of boiling water and extracted for 15 mins. The solution was decanted and the residue ground in a mortar and re-extracted with 10 ml. of boiling water for a second 15 mins. interval. The solution was again decanted and the residue placed in a test-tube and extracted on a boiling water-bath with three successive portions of 10 ml. of hot water, taking 30 mins. for the final extraction. The residue was strained with pressure through a piece of cheese cloth. The total cooled extract was made up to 60 ml. The frozen liver was extracted in exactly the same manner as the muscle tissue, except that the residue was separated by centrifuge.

When an examination of the whole animal (*minus* intestinal contents and urine) was required, the frozen carcass was minced, placed in 100 ml. of boiling water and extracted for 15 mins. The solution was centrifuged and the residue re-extracted with three successive portions of boiling water (100 ml.), the last extraction interval being 30 mins. The cooled extract was made up to 500 ml.

To 60 ml. of the tissue extract were added 15 ml. of 10 % $ZnSO_4, 7H_2O$ solution and 15 ml. of 0·5 N NaOH solution, gradually with shaking of the flask. The precipitates from the muscle and liver extracts were filtered, using an ordinary funnel. The whole animal filtrate was obtained by suction through a Büchner funnel of wide area. It was later found more convenient to precipitate 120 ml. of the whole animal extract, especially in the case of larger animals, as the filtration was slow owing to the presence of fat. In such cases there was occasionally some difficulty in obtaining a clear filtrate. 4·5 g. of purified Lloyd's reagent were added to 50 ml. of the zinc filtrate and the mixture shaken for 2 mins. and filtered. It was found necessary to make this filtrate alkaline with two drops of 40 % NaOH solution to precipitate the excess zinc present. Any excess zinc was found to interfere with the sugar oxidation and also with the removal of the sugars by yeasts. The precipitate of $Zn(OH)_2$ was filtered off, and the filtrate was adjusted to p_H 6–6·5, with 10N H_2SO_4.

The above details form a slight modification of Somogyi's [1930] directions for the preparation of blood-filtrates suitable for sugar analysis. We were unable to prepare suitable filtrates from tissue by the use of tungstic acid [Power and Clawson, 1928].

Analysis for glucose and galactose. The final clear filtrates from the alimentary tract, muscle, liver or whole animal were analysed for glucose (fermentable sugar) and galactose by the method of Harding and Grant [1931–32]. A double action of ordinary baker's yeast and galactose-acclimatised yeast[1], followed by a double centrifugation previous to the determination of the reducing power, ensured the complete removal of the desired sugar at each stage of analysis. The reducing power was determined by a modified Shaffer-Somogyi reagent [Harding and Downs, 1933]. Galactose in the blood specimens was determined by the method of Grant and Glaister [1933] which is suitable for the estimation of non-fermentable sugar in blood. This method of determining galactose in presence

[1] The authors wish to thank the Standard Brands Inc. New York for the continued preparation of "galac" yeast.

of glucose and the reducing substances in blood corpuscles has been later employed by Harding *et al.* [1933] in the determination of blood-xylose. Urine was analysed according to the previous directions of Harding and Grant.

Recovery of added galactose. Galactose added to muscle, liver or whole animal hash was recovered in 98–105 % yield; added to intestinal contents the recovery yield was 80–90 %.

Results and Discussion.

The concentrations of glucose, galactose and residual reducing substances together with the total amounts of galactose found in the alimentary tract, urine and whole animal are shown in Tables I (Series A) and II (Series B).

Table I. *Determinations in whole animals of concentrations of tissue "sugars" in individual control rats, and average galactose values at stated intervals after the ingestion of approximately 0·6 g. galactose per kg. body weight (Series A).*

Rat No.	Rat Wt. (g.)	Dose mg.	Time mins.	Blood-galactose mg./100 ml.	Tissue "sugars" (mg./100 g.) Glucose	Galactose	Residual reduction (as glucose)	Galactose distribution. Total amount in mg. (as galactose) Alimentary tract	Urine	Tissues (as free sugar)	Utilised
51	—	—	—	—	60	4	30	—	—	—	—
52	—	0	0	0	38	2	18	0	—	—	—
53	—	—	—	—	41	(1)	18	—	—	—	—
54	—	—	—	—	55	(1)	24	—	—	—	—
				Average	48	2	22				
Average of 4	143	92	5	0	39	3	19	76	—	6	10
" 7	162	90	20	77	41	19	20	40	2	29	17
" 8	153	89	60	44	44	25	23	4	15	34	36
" 5	147	94	180	11	49	3	24	0	25	4	64

Table II. *Average concentrations of tissue "sugars" and total amounts of galactose in whole animals after ingestion of approximately 1·2 g. galactose per kg. body weight at end of stated intervals (Series B).*

No. of exps.	Average Rat wt. (g.)	Average Dose mg.	Time mins.	Blood-galactose mg./100 ml.	Tissue "sugars" mg./100 g. Glucose	Galactose	Residual reduction (as glucose)	Galactose distribution. Total amount in mg. (as galactose) Alimentary tract	Urine	Tissues (as free sugar)	Utilised
6	156	184	20	86	39	21	16	119	3	32	30
6	134	180	60	126	49	63	19	12	42	82	45
5	148	185	120	57	47	23	21	3	75	34	73
6	150	191	180	20	58	6	20	(1)	76	9	105

At the outset we wish to emphasise the relatively small dose of galactose we have given. Series A received an average of 0·6 g. per kg. and Series B, 1·2 g. per kg. These amounts are comparable with the feeding of 40 and 80 g. galactose to the adult man. True, even these amounts are much in excess of what either the rat or man would receive under natural conditions, but they are much less than those administered by Cori [1925], who gave 8–9 g. per kg. by mouth to the rat, or the 2 g. per kg. per hour given intravenously by Wierzuchowski *et al.* [1931] to the dog. The dose given by Cori to the rat is equivalent to 500–600 g. galactose given to man.

Absorption from the intestine. Both in Series A and B, the absorption of galactose is practically complete at the end of an hour. Galactose is known to be rapidly absorbed in the dog [Nagano, 1902], cat [Hewitt, 1924], rabbit [Hédon,

1900], and rat [Cori, 1925]. Mainly from a study of glucose absorption, but stating that the figures on the absorption of other sugars support the same conclusion, Cori has formulated laws of absorption of sugars from the intestine. "The rate of absorption of a sugar is independent of the amount or concentration of the sugar fed." These conclusions for the absorption of glucose have been questioned by other observers [MacKay and Bergman, 1933]. Cori's absorption rate for galactose is 1·96 [1925] and 1·82 [1926] g. per kg. per hour. From his average figures it can be seen that at the end of 3 hours there were left unabsorbed 1·31, 3·47 and 3·94 g. galactose per kg. body weight. Only the first is comparable with our highest amounts fed in Series B. In Table III we show the

Table III. *Rate of absorption of galactose over short periods of time when given in moderate amounts.*

	After 20 mins. Amount absorbed		After 60 mins. Amount absorbed
	mg./100 g.	mg./100 g. per hr.	mg./100 g. per hr.
Series A	31	93	55
Series B	42	125	125

absorption rates (calculated from the data of Tables I and II) of galactose when administered in amounts below those investigated by Cori. In no case does the absorption rate approach 1·82 g. per kg. per hour. Our figures are few but the most significant give a value of 1·25 g. per kg. per hour. The very short time of our absorption periods, however, may introduce an error tending to low results, but in Series B the rate in the first 20 minutes is the same as in the next 40 minutes. Other differences in the experimental conditions are to be noted. Our animals were starved for 24 hours previous to the feeding, Cori's for 48 hours. By analogy with the absorption of glucose and fructose this should raise our rates of absorption rather than decrease them [Cori and Cori, 1927]. Amytal anaesthesia also lowers the rate of absorption about 30 % [Cori, 1931]. Only a few minutes elapsed, however, between the administration of the amytal and the excision of the alimentary tract. Nor did we divide the sugar remaining in the alimentary tract between the stomach and intestine. Any galactose left in the former would of course raise the calculated rate of absorption from the intestine. McCance and Madders [1930] and Trimble *et al.* [1933] have pointed out the part taken by the pyloric discharge in determining rates of intestinal absorption. The subject of absorption of sugars at low initial concentrations invites further investigation.

Fermentable sugar was also found in irregular amounts in the intestinal contents. It is to be noted that both in Series A and B, at the end of 3 hours, when galactose was still present in the blood stream, none was present in the alimentary tract.

Galactose in the tissues. In our control animals the examination of individual muscle or whole animal always showed the presence of small amounts of substance removable by "galac" yeast. This was not removable by the two previous treatments with ordinary baker's yeast, and while the first treatment with "galac" yeast always effected a small removal the second treatment failed to show any further removal. The amounts varied from 1 to 6 mg./100 g. tissue or muscle (Tables I and IV). Such amounts are just at, or very slightly above, our experimental error. Consequently we cannot place too much reliance on them. Liver extracts showed no evidence of such a removal, but these extracts were much more dilute. No free galactose was found in control rat blood or urine,

Table IV. *Concentrations of muscle and liver "glucose," galactose and residual reducing substances one hour after ingestion of 190 mg. galactose by the rat.*

	mg./100 g.					
	Muscle			Liver		
	Glucose	Galactose	Residual reduction (as glucose)	Glucose	Galactose	Residual reduction (as glucose)
Controls	25	0	4	—	—	—
	34	0	5	—	—	—
	26	2	4	101	0	21
	32	6	8	114	0	19
	38	3	7	162	0	20
	33	2	5	97	0	21
	22	2	9	80	0	17
Average	30 ±6	2·5 ±2	6 ±2	111 ±20	0	18 ±3
One hour after galactose	34	33	7	98	104	17
	33	27	14	129	129	25
	35	36	10	144	111	20
	38	33	9	97	130	35
	33	37	5	143	98	21
Average	35 ±2	33 ±4	9 ±3	122 ±21	114 ±14	23 ±7

nor did Harding and Grant find any in normal human blood and urine. However, the dilutions of 1 : 10 used by them would fail to show amounts less than 2–3 mg./100 ml. In view of the fact that free galactose is readily excretable both in man and the rat it seems unlikely that our analytical finding represents galactose as a free constituent of living muscle.

Corresponding to the rapid absorption of galactose from the intestine there is a rapid rise of this sugar in the blood and tissues. Our observations are too infrequent for us to do more than state that the maximum concentration of galactose in the tissues and blood occurs somewhere within or about the first hour, with either the smaller or larger dose. This same maximum in the blood occurs in man. Are we to infer that the absorption of galactose from the intestine of man is as rapid as in the rat?

At the end of 3 hours after the smaller dose, the galactose content of the whole animal has fallen to the low indefinite figure of the control. After the larger dose, it is sufficiently low (4–9 mg./100 g.) to enable us to state that all but traces of the ingested sugar have disappeared.

Fermentable sugar, residual reducing substances and glycogen after galactose. Our method of analysis gives us, in addition to galactose, the concentration of glucose (fermentable sugar) and residual reducing substances. Kosterlitz and Wedler [1933] examined the sugar of the liver after galactose ingestion. They found slight increases in fermentable sugar but not in such amount as to lead to any definite conclusion. Their residual reducing fraction, which included galactose, was increased. Examination of our whole animal experiments shows a slight average increase in "glucose" at the end of the higher dose experiments, but in view of the large variations little importance can be attached to the figures. Harding and Grant found increases in blood-glucose in man after galactose feeding. The normal variations in blood-glucose are small, however, and experimental fluctuations are thus more easily ascertained. Certainly, in the rat, there occurs no transformation of ingested galactose into glucose sufficiently rapid to cause an accumulation of this latter sugar. In an endeavour to check more strictly the possible occurrence of rapid galactose-glucose conversion, we

examined a section of leg muscle and the liver, one hour after the feeding of the higher dose of galactose. The results are shown in Table IV. The increase in the concentration of galactose is plain in both muscle and liver; but again, the big variation in the "glucose" prevents any definite conclusion. Our figures for "fermentable" sugar in rat muscle (30 ± 6 mg./100 g.) are much higher than those recently published by Cori *et al.* [1933] (10–12 mg./100 g.), though these workers obtained much higher figures for the resting muscle of the amytalised animal. They agree more nearly with those of Power and Clawson [1928] and Bischoff and Long [1932]. The occurrence of any extensive glycogenolysis would impair the value of any experiments designed to measure an increase of that sugar after galactose feeding. Our animals were extremely quiet, however, and our muscle freezings and excisions were rapid. It is interesting to note that our figures for the non-fermentable sugar fraction of muscle extract obtained with $Zn(OH)_2$ as a precipitating agent followed by Lloyd's reagent are lower (4–9 mg./100 g.) than those of Cori *et al.* [1933] (8–22 mg./100 g.), who used $HgSO_4$–$BaCO_3$, though two of our animals after galactose gave 10 and 14 mg./100 g.

The constancy of the concentration of residual reducing substances indicates the thoroughness of our methods of extraction and sugar analyses. It indicates also the improbability of any conversion of galactose into another extractable reducing carbohydrate at such a rate that accumulation occurs. It may be noted that Bischoff and Long [1932] claim to have proved the absence of hexose-phosphate from muscle extracts precipitated by $Zn(OH)_2$. Harding and Grant noted also in man the absence of any new reducing carbohydrate in blood and urine after administration of galactose. Bierry *et al.* [1932] have claimed that they have proved the occurrence in liver of a galactose-containing polysaccharide. We did not examine the hydrolysis products of our animal or muscle hash after extraction to see if any combined or non-extractable galactose occurred. We, however, examined both liver- and muscle-glycogen in 2 rats 4 hours after the ingestion of 400 mg. galactose. The glycogen was extracted by the modification of Pflüger's method detailed by Good *et al.* [1933]. The glycogen obtained was hydrolysed and analysed for glucose and galactose. Only glucose was found.

Excretion into the urine. At the end of 20 minutes small amounts of galactose were found in the urine in both Series A and B. At the end of 3 hours 25 % of the smaller dose of galactose was found in the urine and 40 % of the larger. Cori [1926] found 60 % excretion in 4 hours. In both our series the excretion into the urine is greater than in man; 4 % excretion was the highest noted by Harding and Grant in man after the ingestion of 40 g. galactose, and 8 % after the ingestion of 80 g.

Conclusions.

The essential difference between the rat and man in their behaviour to moderate doses of galactose appears to be in the slower rate of utilisation by the rat. Parallels with other sugars are not wanting—thus the rat utilises intravenous glucose at a much more rapid rate than the dog or man [Cori and Cori, 1927]. The low rate of metabolism of intravenously administered galactose is also seen in the dog (0·2 g. per kg. per hour) [Wierzuchowski, 1931]. From Table II it can be seen that an average of 73 mg. galactose disappears in 2 hours from 150 g. body weight. This means an average utilisation of 0·25 g. per kg. per hour. After the corresponding dose to man Harding and Grant found a disappearance of over 90 % of the galactose in 2 hours—a disappearance rate of approximately 0·5 g. per kg. per hour. Whilst these figures, calculated as they are from experiments varying widely in conditions, must be taken as a very rough

approximation, they nevertheless support the idea that galactose utilisation in man is much better than experimental work on other animals has led us to anticipate. This low rate of change in the rat, coupled with the high rate of absorption and excretion, is responsible, according to Guha [1931], for the failure of this animal to grow or maintain life when galactose is the sole carbohydrate. In man and woman [Duel *et al.*, 1932; 1933] galactose is active as a nitrogen-sparing carbohydrate and an anti-ketogenic body. In this latter capacity it is even claimed that it is superior to glucose, its effect lasting 2 or 3 days. These two extreme statements may well be reconciled by a difference in the rate of transposition in different species.

SUMMARY.

Galactose, fed to rats at levels comparable with those at which it may be administered to man (0·6 g. and 1·2 g. per kg.), is almost completely absorbed from the intestine within an hour.

With both dosages of galactose, all but traces of the free sugar has disappeared from the tissues at the end of 3 hours.

There is proportionately a much larger excretion of the ingested galactose into the urine in the rat than in man. There thus appears to be a lower rate of conversion of galactose into its metabolites in the rat.

No evidence was found indicating the conversion of galactose into glucose or into any other reducing carbohydrate. The glycogen formed after galactose ingestion, either in the muscle or the liver, gives only glucose on hydrolysis.

REFERENCES.

Bierry, Rathery and Magnan (1932). *Compt. Rend. Soc. Biol.* **109**, 809.
Bischoff and Long (1932). *J. Biol. Chem.* **95**, 743.
Cori (1925). *J. Biol. Chem.* **66**, 691.
—— (1926). *J. Biol. Chem.* **70**, 577.
—— (1931). *Physiol. Rev.* **11**, 147.
—— Closs and Cori (1933). *J. Biol. Chem.* **103**, 13.
—— and Cori (1927). *J. Biol. Chem.* **77**, 555.
Duel, Gulick and Butts (1932). *J. Biol. Chem.* **98**, 333.
—— MacKay, Jewel, Gulick and Grunewald (1933). *J. Biol. Chem.* **101**, 301.
Good, Kramer and Somogyi (1933). *J. Biol. Chem.* **100**, 485.
Grant and Glaister (1933). *Can. Chem. Met.* **17**, 7.
Guha (1931). *Biochem. J.* **25**, 1385.
Hamilton (1928). *J. Biol. Chem.* **78**, 63.
Harding and Downs (1933). *J. Biol. Chem.* **101**, 487.
—— and Grant (1931–32). *J. Biol. Chem.* **94**, 529.
—— —— (1932–33). *J. Biol. Chem.* **99**, 629.
—— Nicholson and Armstrong (1933). *Biochem. J.* **27**, 2035.
—— —— and Grant (1932–33). *J. Biol. Chem.* **99**, 625.
Hédon (1900). *Compt. Rend. Soc. Biol.* **52**, 41.
Hewitt (1924). *Biochem. J.* **18**, 161.
Kosterlitz and Wedler (1933). *Z. ges. exp. Med.* **87**, 411.
McCance and Madders (1930). *Biochem. J.* **24**, 795.
MacKay and Bergman (1933). *J. Biol. Chem.* **101**, 453.
Nagano (1902). *Pflüger's Arch.* **90**, 389.
Power and Clawson (1928). *J. Biol. Chem.* **78**, Proc. lvi.
Somogyi (1930). *J. Biol. Chem.* **86**, 655.
Trimble, Carey and Maddock (1933). *J. Biol. Chem.* **100**, 125.
Wierzuchowski (1931). *Biochem. Z.* **230**, 187.
—— Pieskow and Owsianey (1931). *Biochem. Z.* **230**, 146.

XXXV. THE CHEMOTHERAPY OF DERIVATIVES OF HARMINE AND HARMALINE. II.

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(*Received January 3rd, 1934.*)

In the first paper of this series, Coulthard *et al.* [1933] described the preparation of a long series of *O*-alkyl derivatives of harmol and recorded their bactericidal properties. It was shown that the peak compound of the normal alkyl series differed with the test organism, being situated at the butyl compound for *Bacillus typhosus* and at the amyl for *Staphylococcus aureus*, whilst it was recorded briefly that the most effective of the *n*-alkylharmols towards *Entamoeba histolytica* was *O*-*n*-nonylharmol. The experimental results upon which the latter statement was based are described in the present communication.

The organism chosen for our tests was *Entamoeba histolytica*, two strains of which, D.K.B. and N.R.S., were kindly supplied by Mr C. Dobell. Since a survey of the literature appeared to indicate that emetine was the most efficient lethal agent for this protozoon *in vitro*, this alkaloid was used as a control, but in addition tests were made upon some other substances used, or recommended for use, in the treatment of amoebic dysentery.

Laidlaw *et al.* [1928] described a method of testing emetine in which the test organism, *Entamoeba histolytica*, is cultivated in a medium consisting of Ringer's solution with horse-serum and a little rice starch, disodium hydrogen phosphate being added as a buffer. In this medium they found that the amoebae were destroyed in four days by emetine 1 in 5,000,000 provided that the medium did not become too acid. When the phosphate buffer was omitted, however, the end-point was not always sharp but varied from strain to strain and even with the same strain, the tubes which became most acid in culture being most likely to contain living amoebae. In these experiments the lethal concentration varied from 1 in 300,000 to 1 in 750,000.

In order to carry out our tests in such a manner that the minimum lethal concentration of our emetine control was relatively level from test to test, the medium buffered with phosphate was used in a series of determinations of the amoebicidal efficiency of the hydrochlorides of these harmine and harmaline derivatives. The results of this series of tests are given in Table I.

This table shows the gradual increase of the minimum lethal concentration of the *O*-*n*-alkylharmols from 1 in 80,000 (*O*-*n*-ethylharmol hydrochloride) to 1 in 200,000 to 1 in 500,000 for the *O*-*n*-nonyl derivative with subsequent decline for higher members of the series. The corresponding derivatives of harmalol are less efficient as are the *iso*-alkyl ethers of harmol.

The figure obtained for emetine is in good agreement with that (1 in 5,000,000) found by Laidlaw *et al.* [1928] using the same medium. By the courtesy of Dr T. A. Henry we obtained samples of *O*-methylpsychotrine sulphate and conessine acid oxalate, and brief reference to our results with these two salts may

Table I.

Compound	Minimum concentration lethal to *Entamoeba histolytica*
Hydrochlorides of:	
Harmol	1 in 40,000 to 1 in 80,000
O-Ethylharmol	1 in 80,000
O-*n*-Propylharmol	1 in 80,000 to 1 in 120,000
O-*n*-Butylharmol	1 in 20,000 to 1 in 80,000
O-*n*-Amylharmol	1 in 100,000 to 1 in 200,000
O-*n*-Hexylharmol	1 in 100,000 to 1 in 200,000
O-*n*-Heptylharmol	1 in 200,000
O-*n*-Octylharmol	1 in 200,000 to 1 in 300,000
O-*n*-Nonylharmol	1 in 200,000 to 1 in 500,000
O-*n*-Decylharmol	1 in 100,000
O-*n*-Dodecylharmol	1 in 100,000 not lethal
O-*iso*Propylharmol	1 in 80,000 not lethal
O-*sec*-Octylharmol	Less efficient than *O*-*n*-octylharmol
O-Allylharmol	1 in 80,000
O-Benzylharmol	1 in 80,000
Emetine hydrochloride	1 in 2,000,000 to 1 in 10,000,000
Conessine acid oxalate	1 in 80,000 to 1 in 200,000
O-Methylpsychotrine sulphate	1 in 80,000 not lethal

be made in passing. The very low result obtained with methylpsychotrine bears out the clinical results of Jepps and Meakins [1917] and the laboratory work of Dobell and Laidlaw [1926]. On the other hand, the figure obtained for conessine appears surprisingly low in view of the work of Brown [1922] and Henry and Brown [1923] on the action of conessine on free living protozoa, and the experiments of Chopra *et al.* [1927] on *Entamoeba histolytica* from kitten's faeces. A number of clinical successes have been reported with kurchi but it is interesting to note that conessine is regarded by some workers as less efficient than kurchi itself. [See, for example, Caius and Mhaskar, 1928; Knowles, 1928; Henry and Brown (quoting Willmore), 1928.] In support of our figure it may be noted that Knowles [1928] as the result of a clinical investigation suggested that the injection of conessine or total kurchi alkaloids was less efficient than kurchi orally, and Leake [1932] also considers kurchi ineffective, quoting the result of a clinical investigation by Reed using kurchi bismuth iodide.

It is evident from various papers, for example, that of Laidlaw *et al.* [1928] and that of Henry and Brown [1923], that the exceedingly high efficiency of emetine *in vitro* of the order of 1 : 5,000,000 is only found in alkaline, neutral or only very faintly acid media. Our results afford abundant confirmation of this fact. When endeavouring to assess the value of an amoebicide in the treatment of amoebic dysentery by comparison with emetine *in vitro* it appears therefore necessary to consider carefully the hydrogen ion concentration likely to be met with in the areas infested with amoebae.

We have been unable to find any reference to the actual hydrogen ion concentration in the amoebic ulcer, but Knowles *et al.* [1923] found that the p_H of a number of stools containing motile amoebae averaged 6·22. They also reported the results of experiments on kittens artificially infected with *E. histolytica*, in which the colon and rectum of the animals were minced in saline and the hydrogen ion concentration of the suspension determined. The average p_H value obtained in these experiments was 6·33, and the livers when similarly treated showed an average p_H value of 6·34.

Furthermore, a considerable amount of work has been carried out upon the reaction of living, dead and diseased body cells, and the work of Rohde [1927, 1, 2],

and Chambers *et al.* [1927] suggests that the contents of the ulcers may have a hydrogen ion concentration more acid than p_H 7·0.

A consideration of these papers suggests that in any comparisons of amoebicides with emetine *in vitro* the effect of acidity should be studied, particularly when the amoebicides are to be administered orally, and that tests should be carried out at a p_H value of 6·2 or 6·3.

We have carried out a number of tests to study the efficiency of emetine at various hydrogen ion concentrations. In the course of this work we noted that the amount of rice starch added to the tubes of culture medium might also considerably influence the results. Table II summarises the results of three tests,

Table II.

Dilutions of emetine hydrochloride	Test A Medium without 0·2 % phosphate R.S. 0·02 g. p_H 7·7	Test A Medium without 0·2 % phosphate R.S. 0·08 g. p_H 7·57	Test B Medium without 0·2 % phosphate R.S. 0·02 g. p_H 7·15	Test B Medium without 0·2 % phosphate R.S. 0·02 g. p_H 6·2 about	Test C Medium with 0·2 % phosphate R.S. 0·02 g. p_H 6·65	Test C Medium with 0·2 % phosphate R.S. 0·02 g. p_H 6·22	Test C Medium with 0·2 % phosphate R.S. 0·02 g. p_H 6·12
1–1,000,000	O	AA	O	AA	AA	.	AA
1–750,000	.	.	.	.	AA	AA	AA
1–600,000	O	A	O	AA	.	.	.
1–500,000	.	.	.	.	AA (6·57)	AA	AA
1–400,000	O	O	.	AA	.	.	.
1–300,000	.	.	.	AA	O (6·89)	AA (6·53)	AA
1–200,000	O	O	.	AA (5·57)	O (6·57)	O (6·60)	AA (5·97)
1–100,000	.	.	.	AA (5·83)	O (6·46)	O (6·73)	AA (6·33)
1–50,000	.	.	.	.	.	O (6·71)	O (6·49)

Medium = Horse-serum + Ringer's solution.
R.S. = Rice starch.
AA = Living amoebae in original culture.
A = Living amoebae in subculture only.
O = No amoebae observed in original culture or in subculture.

two in medium without phosphate showing the effect of increased acidity and of increased rice starch, and one in medium with buffer showing the effect of increased acidity. Approximately equal amounts of rice starch were added to each tube in a series by preparing a suspension of sterile starch in sterile Ringer's solution and adding equal volumes of this aseptically. The p_H value of this culture medium varies with the methods of determination. The figures quoted were obtained by Mr E. A. Harvey, using the potentiometer and the quinhydrone electrode, those at the top of each column refer to the unsown medium and those inserted in the column to the sown medium after incubation.

If we now proceeded to compare our peak compound, *O-n*-nonylharmol, with emetine under those conditions of acidity which it is suggested may represent conditions *in vivo* more nearly, the ratio of efficiencies showed a considerable alteration (Table III).

Table III.

Culture medium	Starch (per tube) g.	p_H before sowing	p_H after incubation	Lethal concentration: *O-n*-Nonylharmol hydrochloride	Lethal concentration: Emetine hydrochloride
With phosphate 0·2 %	0·02	6·18	6·2	1–300,000	1–400,000
,,	0·02	6·65	6·46 to 6·89	1–200,000	1–300,000
,,	0·02	6·22	6·53 to 6·73	1–100,000	1–200,000
,,	0·02	6·12	5·97 to 6·49	1–100,000	1–50,000
No phosphate	0·02	6·5	—	1–200,000	1–100,000
,,	0·08	7·4	—	1–100,000	1–100,000

After incubation the hydrogen ion concentration of several tubes was determined, and the limits between which these tubes fell are given in column four. Similar results are obtained if unbuffered medium be used.

It has thus been demonstrated that the amoebicidal value of *O-n*-nonylharmol hydrochloride, although considerably less than that of emetine under optimum conditions for this compound, may nevertheless equal, or even surpass, that of the latter if these conditions are varied slightly, particularly if the reaction of the medium is allowed to become more acid. It has also been pointed out that the literature indicates the possibility that the hydrogen ion concentration in the gut may be in the neighbourhood of p_H 6·2 in cases of amoebic dysentery. It therefore appeared desirable that clinical comparison of *O-n*-nonylharmol hydrochloride with emetine should be carried out, and this substance has therefore been submitted to the Therapeutic Trials Committee of the Medical Research Council with this end in view.

It may also be noted that additional confirmation of the amoebicidal value of *O-n*-nonylharmol is given by Gunn [1932]. Experimenting with *Amoeba proteus*, he found it lethal in 24 hours at a dilution of 1 in 1,000,000 or even 1 in 2,000,000.

SUMMARY.

1. The members of a long series of *O*-alkyl derivatives of harmol have been tested for amoebicidal efficiency *in vitro* in comparison with emetine by the method of Laidlaw, Dobell and Bishop.

2. The most efficient derivative, *O-n*-nonylharmol, has been compared with emetine under different conditions. The results indicate that *O-n*-nonylharmol may be equal to, or even better than, emetine as an amoebicide under appropriate conditions.

I have great pleasure in thanking Mr C. Dobell for his advice when we were about to commence this work and for providing strains of amoebae, Dr F. L. Pyman for general direction of the investigation, Dr T. A. Henry for supplying the *O*-methylpsychotrine sulphate and conessine acid oxalate, and Miss D. Wainman for much careful work at the bench.

REFERENCES.

Brown (1922). *Brit. Med. J.* i, 993.
Caius and Mhaskar (1928). Indian Research Memoir, through *Lancet*, i, 38.
Chambers and Pollack (1927). *J. Gen. Physiol.* **10**, 739.
Chambers, Pollack and Hiller (1927). *Proc. Soc. Exp. Biol. Med.* **24**, 760.
Chopra, Gupta, David and Ghosh (1927). *Ind. Med. Gaz.* **62**, 140.
Coulthard, Levene and Pyman (1933). *Biochem. J.* **27**, 727.
Dobell and Laidlaw (1926). *Parasitology*, **18**, 206.
Gunn (1932). *Quart. J. Pharm.* **5**, 63.
Henry and Brown (1923). *Trans. Roy. Soc. Trop. Med. Hyg.* **17**, 61.
—— —— (1928). (Quoting Willmore.) *Lancet*, i, 108.
Jepps and Meakins (1917). *Brit. Med. J.* ii, 645.
Knowles (1928). *Ind. Med. Gaz.* **63**, 455.
—— Napier and Das Gupta (1923). *Ind. Med. Gaz.* **58**, 151.
Laidlaw, Dobell and Bishop (1928). *Parasitology*, **20**, 207.
Leake (1932). *J. Amer. Med. Assoc.* **1**, 195.
Rohde (1927, 1). *Zentr. Chir.* 2134.
—— (1927, 2). *Deutsch. med. Woch.* **53**, 352.

XXXVI. SEPARATION OF CYSTEINE FROM ASCORBIC ACID BY MERCURIC ACETATE.

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(*Received January 5th, 1934.*)

Different investigators have demonstrated that the reducing capacity of plant juices, determined by means of 2 : 6-dichlorophenolindophenol solution, accounts accurately for its observed antiscorbutic potency [Tillmans *et al.*, 1928; 1932; Birch *et al.*, 1933; Harris and Ray, 1933]. This agreement also has been demonstrated with animal tissues [Harris and Ray, 1932; 1933; Birch *et al.*, 1933]. However, biological tests on Jensen rat sarcoma, carried out by Harris [1933], indicate that other reducing substances must be present, because the antiscorbutic activity is much less than would be calculated from the reduction value.

When the titration is carried out in acid medium as indicated by Svirbely and Szent-Györgyi [1933], Birch *et al.* [1933] and Wolff *et al.* [1933], glutathione does not interfere. The presence of cysteine however will be a source of error in the titration with the indicator, because cysteine also reduces the indicator in acid solution [Birch *et al.*, 1933], though not as rapidly as does ascorbic acid. The quantity of cysteine can be determined [Birch *et al.*, 1933] by means of Sullivan's test [Sullivan, 1929, 1, 2], whereas the reduction of iodine solution in acid medium is a measure of the total amount of reducing substances which are present; but we cannot assume that the difference between the total iodine reduction and the cysteine iodine value (calculated from Sullivan's reaction) is due to ascorbic acid. The indicator solution (0·02 % in water) is added from a burette to the solution to be tested (acidified by dilute trichloroacetic acid) till a permanent faint pink colour is obtained. The titration is finished within a minute, whereas the colour generally does not fade after about half a minute. Sometimes this end-point is not sharp, owing to the presence of slowly-reducing substances, because pure ascorbic acid solutions do not show this tendency. Titrating a solution of cysteine-HCl in this way (containing 4 mg. per ml.), 1 ml. solution (acidified by 2 drops trichloroacetic acid) decolorises about 0·4–0·5 ml. indicator after 1 minute. When however 2 ml. indicator solution at once are added to 1 ml. cysteine solution, decoloration takes place in about half a minute. This different behaviour is mentioned because the titration has also been carried out by others by adding the unknown solution to a definite volume of the indicator [Birch *et al.*, 1933]. The two different methods of titration are without influence upon the amount of indicator used by ascorbic acid solutions.

In a previous communication [Van Eekelen *et al.*, 1933] it was stated that cysteine is precipitated by mercuric acetate, whereas ascorbic acid is reversibly oxidised and can be quantitatively regenerated by hydrogen sulphide [Tillmans, Hirsch and Dick, 1932; confirmed by Johnson, 1933].

The ascorbic acid content of solutions of pure ascorbic acid, lemon juice and orange juice was quantitatively recovered by the mercuric acetate precipitation. The reduction with hydrogen sulphide must be effected in a slightly acid

medium, because otherwise, and especially in alkaline solution, reducing sulphur compounds can be formed. It is also important to mention that mercuric acetate solution removes many impurities, a fact which has proved to be very useful in our spectrographic investigations.

Experimental.

Mercuric acetate solution. A 20 % solution in water was prepared and filtered after one day (some hydrolysis takes place).

Precipitation with mercuric acetate solution. To the solution to be tested (which must be slightly acid, excess of acid being removed by $CaCO_3$ and filtration) the mercuric acetate solution is added drop by drop till the precipitation is finished, the reaction taking place in a graduated centrifuge-tube. Care must be taken to avoid a large excess of mercuric acetate, which dissolves the precipitate more or less. After centrifuging, the solution is treated with H_2S. After filtration the solution is left standing overnight, the H_2S removed by nitrogen (controlled by lead acetate paper) and the titration is then carried out.

Example. To 20 ml. of a dilute orange juice solution (titration value 0·5 ml. indicator per ml. solution) 80 mg. crystalline cysteine-HCl were added (4 mg. per ml.). This solution of ascorbic acid and cysteine decolorises after 1 minute about 0·9 ml. indicator per ml. solution (adding the indicator drop by drop to the solution). 18 ml. from this solution (after $CaCO_3$ treatment) required 3 ml. mercuric acetate solution and the procedure given above was followed.

1 ml. solution required 0·43 ml. indicator, calculated $\frac{18}{21} \times 0{\cdot}5 = 0{\cdot}43$ ml. The sodium nitroprusside test was completely negative.

Summary.

Cysteine, being a source of error in the determination of ascorbic acid by means of 2 : 6-dichlorophenolindophenol solution, is removed quantitatively by mercuric acetate solution.

References.

Birch, Harris and Ray (1933). *Biochem. J.* **27**, 580, 590.
Harris (1933). *Nature*, **132**, 605.
—— and Ray (1932). *Biochem. J.* **26**, 2067.
—— —— (1933). *Biochem. J.* **27**, 303.
Johnson (1933). *Biochem. J.* **27**, 1287.
Svirbely and Szent-Györgyi (1933). *Biochem. J.* **27**, 279.
Sullivan (1929, 1). *U.S. Pub. Health Reports*, **44**, No. 24, 1421.
—— (1929, 2). *U.S. Pub. Health Reports*, **44**, No. 27, 1599.
Tillmans and Hirsch (1932). *Biochem. Z.* **250**, 312.
—— —— and Dick (1932). *Z. Unters. Lebensm.* **63**, 267.
—— —— and Hirsch (1932). *Z. Unters. Lebensm.* **63**, 1.
—— —— and Jackisch (1932). *Z. Unters. Lebensm.* **63**, 276.
—— —— and Reinshagen (1928). *Z. Unters. Lebensm.* **56**, 272.
—— —— and Siebert (1932). *Z. Unters. Lebensm.* **63**, 21.
Van Eekelen, Emmerie, Josephy and Wolff (1933). *Nature*, **132**, 315.
Wolff, van Eekelen and Emmerie (1933). *Act. Brev. Neerl.* **3**, 44.

Note added February 6th, 1934. Ergothioneine, which reduces the indicator in acid solution, is also precipitated by mercuric acetate in acid medium.

XXXVII. THE EFFECT OF GUANIDINE DERIVATIVES ON TISSUE RESPIRATION.

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(*Received November 23rd, 1933.*)

The full review of the problem of tetania parathyreopriva recently published by Thomson and Collip [1932] makes it clear that many points in connection with the biochemical mechanism of the production of tetany remain open to question. Whilst it is universally agreed that the outstanding chemical change to be observed in tetania parathyreopriva is the lowering of the blood-calcium, it is still maintained by some authors that this phenomenon is incidental and is only one result of the general intoxication with guanidine derivatives which they regard as the primary cause of the condition.

There is at least no doubt that guanidine derivatives do produce symptoms of tetany, when administered in amounts so small as to indicate that the effect is a genuine intoxication and is not referable to any gross change such as a shift in the acid-base balance of the blood due to the basic character of the guanidine.

It is evident that any information as to the actual physiological effect of guanidine may be expected to be of use in elucidating the mechanism of the production of tetany by this substance and in helping to solve the further question as to whether intoxication with guanidine has in fact anything to do with tetania parathyreopriva.

The object of the present note is to describe the effect of guanidine derivatives on the respiration of isolated tissues.

Experimental.

The statement of Meyerhof [1921] that guanidine depresses the rate of yeast fermentation was confirmed.

For our own investigation we have used for the most part the brains of pigeons and rats; in addition experiments have been made with rat's liver and muscle and with rabbit's blood and nerve. The guanidine derivatives employed were guanidine hydrochloride and carbonate and methylguanidine hydrochloride.

The oxygen consumption of the tissues in air was measured in Dixon and Elliott's [1930] modification of the Barcroft apparatus, the details of the method being the same as those described in a former paper [Gavrilescu and Peters, 1931] except for the preparation of the material. It has been found that in experiments of this type the fineness and uniformity of division of the tissue are of the greatest importance if concordant results are to be obtained; discrepancies previously observed [*cf.* Gavrilescu and Peters, 1931] are probably to be ascribed to insufficient attention to this point. In the present series the liver, removed immediately after the death of the animal, was forced with a bone spatula through a fine sieve; the muscles were crushed in a Latapie grinder. In this way

the tissues were obtained in a finely divided and homogeneous condition, and duplicate determinations usually showed differences of less than 2 %.

0·1 g. of tissue was suspended in 3 ml. Ringer solution in the shaking-bottle of the Barcroft apparatus; in the case of experiments with guanidine derivatives the latter were added, immediately before placing the apparatus in the thermostat, in the form of 0·1 ml. of a 1·5 % solution; the final concentration of guanidine derivative was therefore 0·05 %. In a few experiments (see Table I) this concentration was increased. No change in p_H resulted from the additions of guanidine derivatives. The results are shown in Table I.

Table I.

Average oxygen uptake in mm.³/g./hr.

	Control	Guanidine	Para-thyrine	Guanidine and para-thyrine	% lowering by guanidine
A. Pigeon's brain	1428	1291			9·6
	1497	1399			6·5
	1516	1468			3·1
	1638	1444	1564		11·9
	1521	1258	1464		17·3
	1336	1239	1286		7·1
	1667	1576 (1592, 1341)*			5·4 (5·0, 19·6)
	1344	1329 (1252, 1053)*			1·2 (6·8, 21·7)
B. Rat's brain	1723	1515	1727	1558	12
	1375	1291	1403		6·1
	1744	1347	1629		22·7
	1610	1476	1621		8·3
	1560	1347	1741	1503	7·2
	1777	1567	1604		11·9
	1476	1287			12·9
	1734	1377†	1604	1420†	20·6
	1763	1427†	1747	1418†	19
	1728	1435†	1687		17
C. Rat's liver	572	536	544	540	6·3
	655	611			6·7
	706	692			—
	755	722			4·4
	974	1076			—
	572	573			—
	570	542			5·0
D. Rat's muscle	703	611			13
	175	222			—
	244	268			—
	253	215			15
	286	215			25
E. Rabbit's blood	149	156	191		—
	245	224	222	229	8·6
	222	207	234	182	7·1
	226	219			3·2
	200	191			4·5
	316	265			16
	349	311			10
F. Rabbit's sciatic nerve	277	207			25

* Guanidine concentrations 0·25, 0·5, 1·2 %.
† Methylguanidine.

In Table II are seen the results of an attempt to perform a similar experiment *in vivo*. Col. 1 shows the oxygen uptakes of the brains of six normal rats and col. 2 those of the brains of three rats killed at the height of the symptoms of tetany induced by intraperitoneal injection of 0·5 g./kg. of guanidine.

Table II.

Oxygen uptake of rat's brains in mm.^{3}/g./hr.

	Normal brains	Brains of animals killed at height of tetany after intraperitoneal injection of 0·5 g./kg. guanidine or methylguanidine
	1723	1283
	1744	1511
	1610	1594
	1734	—
	1763	—
	1728	—
Average	1717	1462

Discussion.

It is clear from Tables I and II that in almost all cases the presence of guanidine derivatives has a significantly depressant action on the oxygen uptake of the tissues examined. It may be noted that the effect is less marked with pigeon's brain than with rat's brain, and this may be correlated with the known fact that the toxic dose of guanidine is about twice as high for pigeons as for rats.

It seems evident therefore that the general toxic effect of guanidine, leading to the production of symptoms of tetany, is to be ascribed in part at least to depression of tissue metabolism, in particular the metabolism of the brain. It is possible that this depression may lead to increased irritability of the respiratory centre and hence to alkalosis owing to excessive pulmonary ventilation.

The few experiments in which parathyrine was added gave no indication of a direct antagonistic action between the parathyroid hormone and the guanidine derivatives; our work does not therefore so far throw any light on the possible connection of guanidine intoxication with tetania parathyreopriva.

Summary.

The oxygen uptake of isolated tissues (brain, liver, muscle) is significantly depressed by the presence of guanidine or methylguanidine in approximately 0·05 % concentration.

The oxygen uptake of the brain of rats poisoned with guanidine is lower than that of normal rat's brain.

We are indebted to Prof. I. Cantacuzino for help with the animal experiments.

REFERENCES.

Dixon and Elliott (1930). *Biochem. J.* **24**, 820.
Gavrilescu and Peters (1931). *Biochem. J.* **25**, 2150.
Meyerhof (1921). *Pflüger's Arch.* **188**, 114.
Thomson and Collip (1932). *Physiol. Rev.* **12**, 309.

XXXVIII. THE DETERMINATION OF AMINO-ACIDS IN WHEAT FLOUR.

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THE work here reported was undertaken as a preliminary to the investigation of the changes in the amino-acid content of flour dough during fermentation. In order to study these changes it is necessary to stop the enzyme action at different times before extracting and estimating the amino-acid content, unless the extract can be prepared in a time negligibly short in comparison with the time that fermentation has proceeded, and provided that there is no production of amino-acid in the aqueous extract. A disadvantage of the freezing method employed by Brownlee and Bailey [1930] to inhibit enzyme action is that the enzymes are not destroyed, and may become active during the extraction if this is carried out at normal temperatures, so that observed results may not necessarily be due to fermentation in the dough.

Denham and Scott Blair [1927] found that the variations in amino-acid content of flour-water suspensions for the first few hours after mixing, which were described by Swanson and Tague [1916; 1917], also occurred in the clear extract of the flour and concluded that the proteolytic activity of a flour could be measured by the increase in amino-acid content of the extract. The method of estimation used by Denham and Scott Blair, however, is open to criticism, as shown later. Swanson and Tague [1916] found that the amino-acid content of a flour extract increased on standing, but as their estimations were made weekly they are not comparable with those of Denham and Scott Blair [1927]. Herd [1931] reported that "the change in amino-nitrogen content of the flour extracts is negligible in all cases over a period of 118 hours," but the change in titre reported by Denham and Scott Blair [1927] would not cause a significant change in the titres reported by Herd [1931], and differences in technique render the two sets of results not strictly comparable.

In view of these apparently conflicting statements it seemed advisable to investigate the change in amino-acid content of a flour extract on standing, particularly for the period immediately after the separation of the flour.

Methods based on the formaldehyde titration of Sørensen [1907, 1] and the alkalimetric titration of Foreman [1920] have been used in flour work, and the Sørensen method has always been found the most suitable, though in the data published by Cairns and Bailey [1928] on a comparison of the two methods, the Foreman titration was carried out in 50 % alcohol, and its rejection was therefore not entirely justified since in 50 % alcohol the polypeptides would titrate but only a part of the amino-acids [see Willstätter and Waldschmidt-Leitz, 1921].

EXPERIMENTAL.

Although the procedures described by Swanson and Tague [1916] (for the Sørensen titration), by Foreman [1920] and by Harris [1923] (for a modified Foreman titration) all gave results accurate to within a few per cent. of the theoretical figures with pure amino-acids, these methods were

found to be unsuitable for flour extracts, the Foreman and Harris methods because they involve the dilution of the extract with nine volumes of 95 % alcohol (and the amino-acid content of the extract is originally very small) and the Swanson and Tague method because the slight opalescence of the flour extract renders the final end-point with phenolphthalein or thymolphthalein somewhat indefinite. The technique recommended by Brown [1923] for the formaldehyde titration of bacteriological media was therefore tried and was found very satisfactory.

To 10 ml. of flour extract was added 0·2 ml. of phenol red solution and the solution titrated to p_H 8·0 with $N/14$ NaOH, using a micro-burette calibrated to 0·01 ml. The end-point was obtained by matching with a buffer solution of p_H 8·0 in a comparator block. This titration (stage 1) represents the titratable acidity of the extract. To the sample were then added 8 ml. of commercial 40 % formaldehyde, 0·18 ml. of indicator solution, and the mixture was again titrated to p_H 8·0. (0·18 ml. of indicator was added because the blank titration on the formaldehyde is about 1 ml.). This second stage titration represents the alkali required both to neutralise the amino-acid in solution and to bring the formaldehyde to p_H 8·0. In each case suitable screens of extract and of diluted extract were used in the buffer side of the comparator block. This second stage titration was corrected by subtracting from the titre a blank titration to p_H 8·0 of 8 ml. of formaldehyde diluted with 10 ml. of distilled water instead of bringing the formaldehyde to p_H 8·0 before adding to the flour extract. No change in the blank titration was ever observed during the few hours occupied by the experiments to be described.

The end-points obtained by this method were very sharp and distinct and were sensitive to less than 0·01 ml. of $N/14$ NaOH. An endeavour to increase the sensitivity of the method by using $N/100$ alkali was not very successful owing to lack of distinctness of the end-point. Other indicators offered no advantage.

It is recognised that, under the conditions of this work, titrating the test solution to a colour match with the standard buffer solution does not necessarily mean that the p_H of the test solution and the buffer solution are the same [see Halton and Fisher, 1928], but the conditions are at least reproducible. Moreover, the electrometric determination of the p_H of a solution containing 16 % of formaldehyde would introduce difficulties in the error of the junction potential with the formaldehyde solution.

Table I. *Amino-nitrogen in flour extract at various times.*

Titration	Time (mins.)	Stage 1, ml. $N/14$ alkali per 2 g. flour	Stage 2, mg. amino-N per 2 g. flour
Flour (*a*)			
1	0	0·21	0·30
2	10	0·21	0·30
3	20	0·21	0·30
4	60	0·22	0·30
5	90	0·22	0·29
6	120	0·21	0·30
7	165	0·21	0·31
8	225	0·21	0·30
9	345	0·22	0·30
10	495	0·21	0·31
Flour (*b*)			
1	0	0·24	0·30
2	10	0·24	0·30
3	25	0·23	0·31
4	40	0·22	0·30
5	75	0·23	0·30
6	120	0·22	0·30
Flour (*c*)			
1	0	0·31	0·41
2	20	0·32	0·42
3	40	0·32	0·41
4	70	0·32	0·41
5	120	0·33	0·41
6	180	0·32	0·41

For the preparation of the extract the method described by Denham and Scott Blair was used. Flour, equivalent to 40 g. dry flour, was extracted by shaking for five minutes with 200 ml. of distilled water to which toluene had been added, the mixture centrifuged and the slightly opalescent extract decanted through a filter. The filtrate was titrated immediately and after various intervals of time. Representative results, given in Table I, for three different flours, (*a*) from a typical mill mixture, (*b*) from English wheat, (*c*) a 30 % low grade flour, show that there was no change in the amino-acid content of the extract on standing in the presence of toluene, nor was there any change in titratable acidity.

Further experiments in the absence of toluene gave the same results, thus confirming the finding of Denham and Scott Blair that for short period experiments toluene did not affect the results. Similar results were obtained when the accuracy of the method was increased five-fold by titrating 50 ml. of solution instead of 10 ml., so that the failure to observe an increase in amino-acid content with time was not due to the insensitivity of the method. Experiments were conducted in which the extract was maintained at 25° and 37° respectively, but no increase in amino-acid titration was obtained.

That the flours used exhibited the phenomenon of increase in amino-acid content with increasing time of extraction is shown in Table II. This table also shows the increase in titratable acidity (stage 1) with increasing time of extraction. This increase is due, at least in part, to an increase in the buffer value of the extract, caused by the production of inorganic phosphate from phytin by the action of phytase [Bailey and Peterson, 1921], but may also be due to the production of organic acids by fermentation processes. The low-grade flour (*c*) shows a much greater increase in amino-nitrogen than flours (*a*) or (*b*).

Table II. *Increase in amino-nitrogen with time of extraction.*

Time of extraction	Stage 1, ml. *N*/14 alkali per 2 g. flour	Stage 2, mg. amino-N per 2 g. flour
Flour (*a*)		
5	0·19	0·30
20	0·27	0·32
35	0·32	0·35
50	0·33	0·35
Flour (*b*)		
5	0·24	0·30
15	0·28	0·33
30	0·33	0·33
55	0·40	0·37
75	0·44	0·38
105	0·49	0·39
Flour (*c*)		
5	0·31	0·42
30	0·56	0·48
60	0·73	0·54
120	0·85	0·55

Since the results shown in Table I are directly opposed to the findings of Denham and Scott Blair, the work was repeated, using the method of estimation described by these authors (an acid titration of one portion to p_H 5·5 with methyl red and an alkali titration of a second portion to "a full rose colour" with phenolphthalein[1] in the presence of formaldehyde) both in the presence and absence of toluene. With the mill mixture and English flours no increase in amino-acid content was obtained either with or without toluene, but the extract of the low-grade flour showed an increase in the titration with time whether

[1] For this end-point a buffer solution of p_H 9·0 was used in the work here described.

Table III. *Amino-nitrogen content of extract of low-grade flour (no toluene).*

	Denham and Scott Blair method			Brown method	
Time mins.	ml. acid $N/14$	ml. alkali $N/14$	Total amino-N mg. per 2 g. flour	Stage 1	Stage 2, mg. amino-N per 2 g. flour
0	0·11	0·77	0·88	0·32	0·41
20	0·11	0·85	0·96	0·31	0·41
40	0·11	0·88	0·99	0·31	0·40
60	0·12	0·90	1·02	0·31	0·41
120	0·14	0·91	1·05	0·31	0·41

toluene were present or not. Some typical figures are given in Table III which show an increase in the amino-nitrogen content of the extract as estimated by the method of Denham and Scott Blair and no increase by the technique of Brown. Table IV shows the absence of any change in amino-nitrogen content of the "mill mixture" and the English flour and the increase in the case of the low-grade flour by the Denham and Scott Blair method irrespective of the presence or absence of toluene.

Table IV. *Amino-nitrogen content of flour extracts with and without toluene.*

	Denham and Scott Blair method					
	Without toluene			With toluene		
Time mins.	ml. acid $N/14$	ml. alkali $N/14$	Total amino-N mg. per 2 g. flour	ml. acid $N/14$	ml. alkali $N/14$	Total amino-N mg. per 2 g. flour
English flour						
0	0·06	0·63	0·69	0·06	0·63	0·69
30	0·06	0·62	0·68	0·06	0·63	0·69
60	0·06	0·64	0·70	0·06	0·63	0·69
120	0·06	0·63	0·69	0·06	0·63	0·69
240	0·06	0·63	0·69	0·06	0·63	0·69
Mill mixture						
0	0·07	0·60	0·67	0·07	0·61	0·68
30	0·07	0·60	0·67	0·07	0·60	0·67
60	0·07	0·61	0·68	0·07	0·61	0·68
120	0·07	0·61	0·68	0·07	0·61	0·68
Low grade flour						
0	0·10	0·79	0·89	0·11	0·80	0·91
30	0·11	0·88	0·99	0·11	0·89	1·00
60	0·11	0·92	1·03	0·12	0·92	1·04
120	0·12	0·94	1·06	0·13	0·94	1·07
180	0·13	0·96	1·09	0·13	0·95	1·08

The slight discrepancies between the amino-nitrogen content of the low grade extract with and without toluene are due to the technique of this experiment which was to prepare several hundred ml. of extract, divide this into two portions and add toluene to one portion. Each portion was then titrated in turn, so that before the initial titration the "toluene" extract had stood a slightly longer period than had the "no toluene" extract, and this is reflected in the slightly higher amino-nitrogen content of the "toluene" extract.

Table III also illustrates the great difference in the absolute magnitude of the amino-acid content as determined by the two methods; that obtained by the Denham and Scott Blair method (the sum of the two titrations) being some two to three times as great as that obtained by titrating to the same p_H before and after the addition of the formalin solution. This discrepancy is due to the titratable acidity and buffer capacity of the flour extract which are included as amino-acid by the Denham and Scott Blair method.

Though the main object of this investigation had been accomplished in showing that no measurable proteolysis occurred in the flour extracts for the few hours immediately subsequent to the separation of the flour, it was thought to be of interest to try and explain the anomalous result obtained with the low-grade flour. Extracts of this flour, prepared as described above, were analysed in the following manner after various periods of standing. 10 ml. of extract were titrated with $N/14$ HCl to p_H 5·5 as in the Denham and Scott Blair method. To another 10 ml. was added 0·2 ml. of thymol blue indicator and the solution titrated to match successively the colours in buffers of p_H 8·0, 8·5, 8·7 and 9·0; formaldehyde and further indicator solution were then added and the mixture again titrated to these end-points. Table V shows the results obtained in one such experiment when toluene water was used for the extraction. The amino-acid by the method of Denham and Scott Blair is the sum of the titration to p_H 5·5, the titratable acidity and the amino-acid by the method of Brown.

From Table V it is seen that there is no change in the buffer value of the solution as shown by the acid titration to p_H 5·5 and the alkali titrations to

Table V. *Determination of amino-acid at various times in the aqueous extract of a low-grade flour by the methods of Brown and of Denham and Scott Blair.*

Time in mins.	from first titration		0	20	40	60	120
	from mixing		20	40	60	80	140
Acid titration to p_H 5·5; ml. $N/14$ HCl			0·10	0·10	0·10	0·10	0·10
p_H 8·0	Titration before adding formaldehyde; ml. $N/14$ NaOH		0·31	0·31	0·31	0·31	0·31
	Amino-nitrogen mg. per 2 g. flour	Brown	0·42	0·41	0·42	0·42	0·41
		Denham and Scott Blair	0·83	0·82	0·83	0·83	0·82
p_H 8·5	Titration before adding formaldehyde; ml. $N/14$ NaOH		0·39	0·38	0·39	0·39	0·38
	Amino-nitrogen mg. per 2 g. flour	Brown	0·38	0·38	0·38	0·38	0·38
		Denham and Scott Blair	0·87	0·86	0·87	0·87	0·86
p_H 8·7	Titration before adding formaldehyde; ml. $N/14$ NaOH		0·41	0·40	0·41	0·42	0·41
	Amino-nitrogen mg. per 2 g. flour	Brown	0·36	0·35	0·35	0·34	0·35
		Denham and Scott Blair	0·87	0·85	0·86	0·86	0·86
p_H 9·0	Titration before adding formaldehyde; ml. $N/14$ NaOH		0·48	0·48	0·48	0·48	0·48
	Amino-nitrogen mg. per 2 g. flour	Brown	0·28	0·33	0·38	0·40	0·48
		Denham and Scott Blair	0·86	0·91	0·96	0·99	1·06

p_H 8·0, 8·5, 8·7 or 9·0 respectively. Also this latter titration indicates that there is no change in the titratable acidity of the extract on standing. The amino-acid by the method of Brown is less the higher the p_H to which the solution is titrated, in accordance with the theory of the titration. At p_H 8·0, 8·5 and 8·7 there is no increase in amino-acid as estimated by either method, but at p_H 9·0 there is an increase by both methods.

It was suspected that this increase at p_H 9·0 was due to the production by enzyme action of substances containing both carboxyl and amino-groups but which did not become titratable even under the action of formaldehyde until p_H 9·0 was reached. If so, such increase might be enhanced were the enzyme

allowed to act on the flour substrate instead of on extracted protein only. A suspension of 40 g. of "dry" flour in 200 ml. of distilled water was allowed to autolyse at 27°. From time to time samples were taken, centrifuged, filtered and the filtrate titrated to p_H 8·0, 8·5, 8·7 and 9·0 respectively as before. The results of one such experiment are shown graphically in Fig. 1, plotting amino-nitrogen against time.

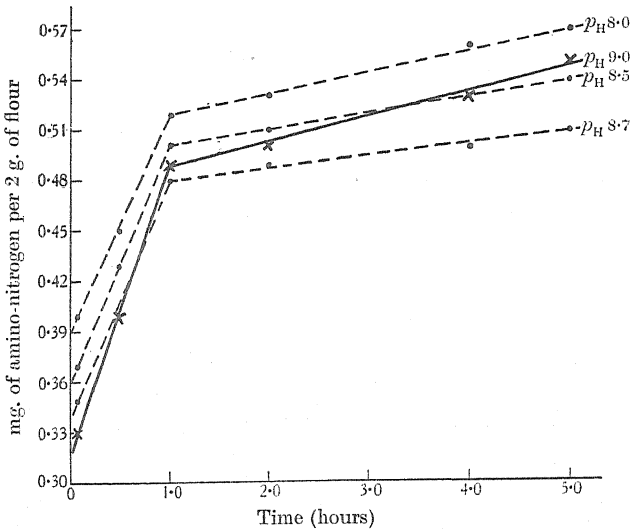

Fig. 1. Amino-nitrogen in flour-water digest at various times.

Initially the amino-acid content is lower the higher the p_H used, as is to be expected, and all values increase with time. This increase is due to further extraction[1] of the amino-acids from the flour and also to the production of amino-acids from the proteins. The rapid increase for about an hour is due almost entirely to extraction, the subsequent slow increase is due to proteolysis, and the slope of this second portion of the graph measures the proteolytic activity of the flour. The graphs at p_H 8·0, 8·5 and 8·7 are practically parallel, but the graph for p_H 9·0 crosses the other graphs, indicating the more rapid production of substances titratable at p_H 9·0.

The addition of yeast to a flour-water suspension did not enhance this effect, because the amino-acid produced was used by the yeast in its metabolism, and this rendered difficult the interpretation of the results from the standpoint of the present work. The indications were, however, that at each estimation after the first a greater titration was obtained at p_H 9·0 than at p_H 8·0 after the addition of formaldehyde. There was also evidence that the titration at p_H 8·7 was increased in the presence of yeast.

Table VI shows the results obtained from a takadiastase digest of the gluten washed from this flour. The amino-nitrogen estimated at p_H 9·0 increases more

[1] It may be noted that extraction was complete in one hour, so that the method of estimating amino-acids in flour given in the Methods of Analysis of the Association of Official Agricultural Chemists, p. 168 (3rd edition, 1930), which requires extraction for a three-hour period, measures not only the amino-acid originally present, but also that produced by some two hours' proteolysis, unless the extraction be carried out at a low temperature.

Table VI. *Amino-nitrogen (mg. per 10 ml. of solution) formed in a gluten-takadiastase digest.*

Time hours	Titration	p_H 8·0	p_H 8·5	p_H 8·7	p_H 9·0
0	1	0·06	0·06	<0·07	<0·08
	2	0·00	0·00	0·00	0·00
1	1	0·10	0·12	0·13	0·14
	2	0·05	0·03	0·02	0·02
2	1	0·12	0·14	0·15	0·16
	2	0·06	0·04	0·06	0·07
3	1	0·14	0·16	0·17	0·18
	2	0·09	0·07	0·09	0·10
4	1	0·16	0·18	0·19	0·21
	2	0·12	0·10	0·12	0·13
5	1	0·18	0·20	0·22	0·23
	2	0·14	0·12	0·14	0·18

Titration 1: initial alkali titration.
Titration 2: alkali titration in presence of formaldehyde less the formaldehyde blank, *i.e.* amino-nitrogen.

rapidly than that at p_H 8·0. In all cases the amino-nitrogen at p_H 8·5 is less than that at p_H 8·0 for the corresponding time in accordance with theory, but it is clear that the substances affecting the estimation at p_H 9·0 are also exercising an influence at p_H 8·7, but to a lesser extent. The action of takadiastase on the gluten is thus comparable with the changes occurring in the flour extract and in the flour suspension.

No such abnormal increase at p_H 9·0 was obtained in pepsin digests of gluten, caseinogen or egg-albumin.

An acid hydrolysis of the moist gluten from this low-grade flour was followed by estimation of the amino-acid from time to time by titration to p_H 8·0, 8·5, 8·7 and 9·0 respectively as before. The results shown in Table VII indicate that hydrolysis was normal, and that the amino-acid estimated at p_H 9·0 is always less than that at p_H 8·0.

Table VII. *Amino-acid formed in acid hydrolysis of flour-gluten.*

Time hours	mg. of amino-nitrogen at p_H 8·0	p_H 8·5	p_H 8·7	p_H 9·0
0	0·00	0·00	0·00	0·00
0·5	0·65	0·62	0·60	0·52
1·0	0·84	0·81	0·80	0·76
2·0	0·93	0·88	0·88	0·82
3·0	1·02	0·98	0·98	0·91
6·0	1·06	1·03	1·03	0·98
9·0	1·09	1·05	1·05	0·98

To determine, if possible, the class of substance responsible for the abnormal increase, various materials probably occurring in the flour-water suspension were titrated to p_H 8·0, 8·5, 8·7 and 9·0 respectively before and after the addition of formalin. Neither protein, peptone nor amino-acid solutions showed any increased titration at p_H 9·0. Indeed all showed the expected decrease in titration with increase in p_H value. These results are shown in Table VIII. All possible mixtures of these solutions were titrated similarly, and the values obtained were strictly additive and gave no evidence of an increased titration

at p_H 9·0 but showed the same reduction in titration as is illustrated in Table VIII. In each case the requisite amount of solution was taken, diluted to 10 ml. and then titrated.

Table VIII. *Amino-acid estimation at* p_H *8·0, 8·5, 8·7 and 9·0 respectively.*

		Phenol red		Thymol blue			
Titration ml. $N/14$ NaOH		p_H 7·0	p_H 8·0	p_H 8·0	p_H 8·5	p_H 8·7	p_H 9·0
Egg-albumin	1	0·03	0·08	0·08	0·09	0·10	0·11
	2	0·21	0·25	0·25	0·24	0·23	0·20
Peptone	1	0·06	0·11	0·11	0·16	0·18	0·20
	2	0·18	0·18	0·18	0·15	0·14	0·09
Alanine (theory 0·91 ml.)	1	0·01	0·02	0·02	0·06	0·09	0·12
	2	0·74	0·89	0·89	0·87	0·86	0·81
Glutamic acid hydrochloride (theory 0·91 ml.)	1	1·82	1·83	1·83	1·89	1·92	2·01
	2	0·57	0·88	0·88	0·83	0·81	0·76

Titration 1: solution without formaldehyde.
Titration 2: difference between titrations before and after the addition of formaldehyde corrected for the blank titration, *i.e.* titration due to amino-acid.
Owing to the large titration to neutralise the solution of glutamic acid hydrochloride, extra indicator was added to obtain the same indicator concentration as in the buffer solution. Also the formaldehyde blank was determined on 8 ml. of formalin diluted with 12 ml. of distilled water.

Discussion.

The results obtained in this study of the estimation of amino-nitrogen in wheat flour show that extraction is complete in about one hour, and that no further formation of amino-acid occurs in the extract after separation from the flour. In the case of one flour, a low-grade, there was an apparent increase in amino-nitrogen in the extract when the estimation was carried out at p_H 9·0, but no increase at the lower p_H values used.

This increase is not due to more complete neutralisation of the amino-acids at the higher p_H, for the more recently published titration curves for individual amino-acids in the presence of formaldehyde show that neutralisation is complete at p_H 8·0[1] [Brown, 1923; Harris, 1929]. In Table VIII the low results at p_H 7·0 are due to incomplete neutralisation of the amino-acid in the presence of formaldehyde, and the low results at $p_H > 8·0$ are due to partial neutralisation of the amino-acids before the addition of formaldehyde. That the maximum titration at p_H 8·0 is slightly less than the theoretical titration value of 0·91 ml. is also due to this latter factor.

Moreover, the facts that in the case of the flour extract, the flour suspension and the takadiastase-gluten digest, lower titrations were recorded at p_H 8·5 than at p_H 8·0, and that there is not a progressive increase in titration with increase in p_H indicate that completion of neutralisation of the amino-acids is not the explanation of the phenomenon.

If it be accepted that the formaldehyde titration estimates carboxyl groups that become titratable because of the action of formaldehyde on the amino-groups, then the observed increase at p_H 9·0 is due to an increase in the number

[1] Since the above was written a recent paper by Van Slyke and Kirk [1933] has been received which shows titration curves of amino-acids compiled from various authors. The titration curves in the presence of formaldehyde show that neutralisation is not complete before p_H 9·0. However, these curves were "estimated from end-points at p_H 9 to 10, given by Sørensen" [1907, 2], and are said to be "only approximately exact" [Van Slyke and Kirk, 1933, p. 657], so that the experimental curves of Brown [1923] and of Harris [1929] are the more reliable.

of carboxyl groups (or amino-groups affected by formaldehyde) which, even in the presence of formaldehyde, do not titrate until p_H 9·0 is reached. That such increase is not due to the production of amino-acids in the usual sense of this term is shown by the titration values at p_H 8·0. Nor is this increase due to the production of proteoses or peptones, as shown by a pepsin digestion, though it occurs in a takadiastase digest of gluten. It is therefore due to substances formed in the enzymic hydrolysis of the proteoses or peptones formed from gluten. A combination of carboxyl and amino-groups in a compound of very low acid dissociation constant may be expected in a substance intermediate between the polypeptides and the amino-acids. Such a substance, under the action of formaldehyde, would not affect the amino-acid estimation at p_H 8·0 because of its very low acid dissociation constant but would become titratable at p_H 9·0. Then in the flour extract, if the proteins present are broken down, not to their constituent amino-acids but to some slightly larger grouping of a few amino-acids, this would be estimated at p_H 9·0 and not at p_H 8·0 and would explain the experimental results.

Although only one of the four flours examined to date has shown this phenomenon of increase in amino-nitrogen at p_H 9·0 and not at p_H 8·0, it seems from the work of Denham and Scott Blair and of Halton (unpublished) that it may be of more common occurrence than this would indicate.

In considering the question of protein changes in a flour dough it is necessary to take into account any such change as is indicated in this paper, for the physical properties of the dough will be affected by any protein degradation whether the decomposition be carried as far as the amino-acid stage or not.

Perhaps it should be emphasised that titration to p_H 9·0 before and after the addition of formaldehyde is not advocated as a method of estimating amino-acids, but that in conjunction with an estimation at p_H 8·0 it may yield some information not afforded by the amino-acid estimation alone.

SUMMARY.

1. The claim that proteolysis occurs in flour extracts has been studied for the few hours immediately subsequent to separation of the extract.

2. The technique of Brown for the estimation of amino-acids by the Sørensen method has been applied to flour extracts.

3. Proteolysis, as measured by the production of amino-acids, did not occur in the flour extracts studied.

4. For one flour of the four studied (a low-grade) the amino-acid determinations made on the extract at varying p_H values showed an increase with time if the solution were titrated to p_H 9·0 before and after the addition of formaldehyde, but no increase if the titration were carried to p_H 8·0 only.

5. It is suggested that this is due to the enzymic production of substances containing carboxyl and amino-groups and having a low acid dissociation constant, which do not titrate even in the presence of formaldehyde until p_H 9·0 is reached.

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REFERENCES.

Bailey and Peterson (1921). *J. Ind. Eng. Chem.* **13**, 916.
Brownlee and Bailey (1930). *Cereal Chem.* **7**, 487.
Brown (1923). *J. Bact.* **8**, 245.
Cairns and Bailey (1928). *Cereal Chem.* **5**, 79.
Denham and Scott Blair (1927). *Cereal Chem.* **4**, 58.
Foreman (1920). *Biochem. J.* **14**, 451.
Halton and Fisher (1928). *Cereal Chem.* **5**, 445.
Harris (1923). *Proc. Roy. Soc. Lond.* B **95**, 500.
—— (1929). *Proc. Roy. Soc. Lond.* B **104**, 412.
Herd (1931). *Cereal Chem.* **8**, 145.
Sørensen (1907, 1). *Biochem. Z.* **7**, 45.
—— (1907, 2). *Compt. Rend. Lab. Carlsberg*, **7**, 1.
Swanson and Tague (1916). *J. Amer. Chem. Soc.* **38**, 1098.
—— —— (1917). *J. Amer. Chem. Soc.* **39**, 482.
Van Slyke and Kirk (1933). *J. Biol. Chem.* **102**, 651.
Willstätter and Waldschmidt-Leitz (1921). *Ber. deutsch. chem. Ges.* **54**, 2988.

XXXIX. A HORIZONTAL MICRO-BURETTE.

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In titrations where we are restricted to determining minute quantities it is necessary to keep the final volume of fluid as low as possible, for in this way we decrease the amount of added reagent necessary to produce an observable change in concentration around the end-point and so minimise the total error. The accurate delivery of small volumes needs special burettes. With the Bang 2 ml. burette the percentage error of delivering 2 ml. is greater than the percentage error of delivering 50 ml. with the ordinary burette. Expressed as a coefficient of variation the ratio is in fact 0·11 to 0·05. Owing however to the large reduction of volume, and provided we are dealing with quantities below a certain level (about 10^{-5} equivalent), increased accuracy is secured with the micro-burette, the advantage being greater the smaller the quantity. At the same time the use of standard solutions ten times more concentrated—and hence in general more stable—is an important added advantage.

It is occasionally required to titrate quantities so minute that a burette delivering volumes of the order of 0·1 ml. is necessary. If the principle of delivering from a vertical burette is retained the delivery error alone rises to 0·45—expressed as a coefficient of variation. In Rehberg's burette [Rehberg, 1925] this delivery error appears to be much lessened by the principle of expelling the fluid by a mercury column controlled by a screw. The percentage error of delivery however can be reduced to about one-fourth the above figure by the simple expedient of delivering from a horizontally placed graduated tube. The accuracy obtained is as great as in Rehberg's burette but the ease of construction and cleaning is much greater. The percentage error of delivering 0·1 ml. with the horizontal burette here described is practically identical with the error of delivering 1 ml. with the Bang 2 ml. burette. It can therefore be substituted for the Bang burette over the whole range of micro-work. It titrates minute quantities (of the order 10^{-6} equivalent, or less) with substantially greater accuracy and permits the use of fluids ten times more concentrated and under better conditions of security from contamination by the carbon dioxide of the air or by alkali from glass surfaces—the delivered fluid having been in contact only with pyrex or paraffined walls.

Description of the burette.

Fig. 1 illustrates the structure of the burette. *A* is a piece of thermometer tubing of approximately 1 mm. bore graduated in centimetres and millimetres and placed horizontally. It is joined by a small length of pressure tubing to tube *B*. To *B* is attached the delivery tube and tap *C* and also the connecting tube and tap *D* communicating with the small container *E* on a hinged platform with support. Behind tap *D* a small window is cut in the wooden supporting

frame to allow free manipulation. All tubing except the graduated thermometer tubing (and this also if possible) should be made of pyrex glass. An advantage may be secured if the tube adjoining the graduated tube has twice or more the internal bore of this latter for then the volume of fluid in the graduated tube will be small in comparison and the emerging fluid will have been in contact only with the pyrex glass or the paraffined walls of the container. The cleansing required for a smoothly running meniscus is very easily done by detaching the graduated tube and cleaning by any of the ordinary methods. It may as a rule be cleaned sufficiently well using alcohol and ether. After cleansing it is replaced with the glass ends in contact and washed out with a

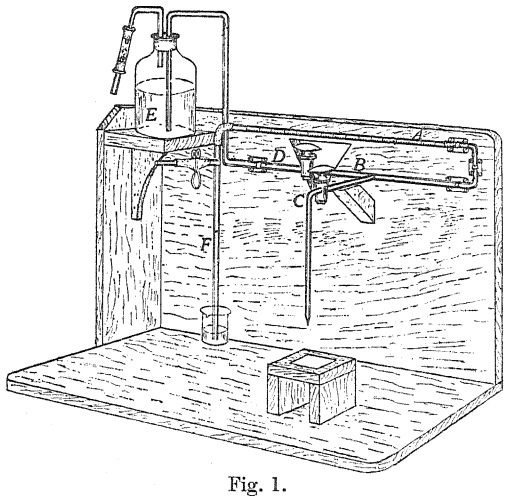

Fig. 1.

few drops of fluid from the container. The small hydrostatic pressure (5, 10 or more cm.) ensures a uniform delivery without drainage owing to the horizontal position. The rate of delivery may be found inconveniently rapid unless the delivery tip is very finely constricted. It is then liable to become blocked by any small solid particle. A simple and efficient method of adequately controlling the full delivery rate with open tap so that fine adjustments of the meniscus may be made on turning consists in attaching a glass tube to the end of the graduated section as in Fig. 1 (*F*). This dips into water below, contained in—say—a 25 ml. beaker. By sucking up some water in this tube and attaching the clip, the delivery pressure of the burette is diminished and controlled as desired.

The use of good quality thermometer tubing will ensure uniformity of bore and lengths of 25 or 50 cm. may be used as desired. With an internal bore of 1 mm. approximately 128 divisions on the burette correspond to a volume of 0·1 ml. For the container a 100 ml. reagent bottle—paraffined inside when weak alkalis are used will be found suitable.

The vertical length of the delivery tube may be varied to suit the particular fluid containers used, whether small beakers, centrifuge-tubes or the special absorbing apparatus described in a previous communication [Conway, 1933]. A standard length of about 10 cm. will suit nearly all containers. Along this delivery tube a fine pyrex tube may be attached by an elastic band or bands at the top and connected with an air-blower for stirring when centrifuge-tubes

or other such containers are used. The tip of the delivery tube may be immersed in the fluid to be titrated or immersed after the emergence of some fluid. If this method is not used a fine pyrex rod will be found suitable for removing small quantities of fluid from the tip and an amount representing a millimetre division on the graduated tube may be quite easily controlled in its emergence and transference to the titrated fluid.

The standardisation of the burette can be carried out by weighing the total of five or more deliveries of the graduated section with subsequent reference to correction tables for transferring grams to millilitres [*e.g.* those given by Stott, 1928]. A titration method may be also used. In the actual use of the burette it is however rarely required to know the volume delivered in ml., the length of the fluid column in the graduated tube corresponding on titration to a measured quantity of standard fluid being taken as the reference figure. This has the advantage of standardising at the same time the fluid in the burette.

To illustrate the action of the burette the variable error of delivering 0·1 ml. will be considered as well as a series of titrations of $10^{-3.7}$ milli-equivalents of sulphuric acid.

The variable error of delivering 0·1 ml. expressed as a coefficient of variation.

This error was investigated by a titration method. Deliveries of 0·1 ml. of N sulphuric acid represented by 128 mm. on the graduated tube were made into a small pyrex dish. The deliveries were successively titrated with $N/10$ carbon dioxide-free alkali from a 2 ml. standard Bang burette, a little indicator mixture of methyl red and methylene blue being added. A similar series of titrations was carried out where 1·0 ml. of $N/10$ sulphuric acid was delivered from another standard 2 ml. burette. The amount of acid titrated in both series was therefore the same and the amount of alkali necessary to change the colour of the indicator of negligible quantity owing to the strength of the solutions. The only difference affecting the variable error between the two sets is that arising from the delivery of 0·1 ml. from the horizontal burette and of 1 ml. from the Bang burette. The difference of the squares of the coefficients of variation for the two sets of titrations is therefore the difference between the squares of the coefficients for delivering these volumes. The coefficients of variation for the two sets were 0·26 and 0·25 respectively. Also the coefficient of variation for delivering 1 ml. from the Bang burette was found from 20 successive weighings to be $0{\cdot}11 \pm 0{\cdot}02$ so that it may be readily calculated that the coefficient for the horizontal burette delivery of 0·1 ml. is 0·12. Subsequent and similar series gave 0·10 and 0·15, so that 0·12 may be taken as the approximate coefficient of variation for delivering 0·1 ml. with the burette described here.

The variable error of titrating 1 ml. of 0·0002 N *sulphuric acid illustrating the minimum variable error of acidimetric titration.*

The object of determining this error is to ascertain the least amount of alkali or acid which can be discriminated in practice by titrating with the burette. If we consider the titration of 1 ml. of $N/10$ acid delivered for example from a 1 ml. simple pyrex pipette (drawn out tube—length 17·5 cm.) the coefficient of variation in millimols of KOH is 25×10^{-5}. Where $N/100$ fluids are used the standard deviation in millimols is ten times less, and further reduction in the strength of the fluids will progressively reduce the standard deviation expressed in absolute units. We reach a point however when the standard deviation so

expressed becomes constant. This limit of standard deviation sets the limit of our discrimination and is proportional to the total buffering power of the fluid at the end multiplied by the indiscernible range here of the p_H figure. The limit is reached for the present horizontal burette when the quantity of acid titrated is in the region of 10^{-4} millimol.

Table I shows the first seven of a series of 20 titrations of 1 ml. of acid-indicator mixture of strength 0·0002 *N* with 0·001 *N* carbon dioxide-free alkali. The acid was of a kind previously described [Conway and Byrne, 1933] containing 20 % alcohol, 0·0008 % methyl red and 0·00016 % methylene blue. The standard deviation in millimols was found to be $1{\cdot}7 \times 10^{-6}$. The total quantity of fluid at the end-point was 1·2 ml. contained in about 1 ml.

Table I.

No. of titration	Quantity of acid millimols	Burette reading (0·001 *N* KOH) cm.	Burette reading as percentage of mean reading
1	$10^{-3{\cdot}7}$	25·6	101·2
2	,,	25·2	99·6
3	,,	25·5	100·8
4	,,	25·1	99·2
5	,,	25·3	100·0
6	,,	25·3	100·0
7	,,	25·5	100·8

The above figures give the results of the first seven titrations of a series of 20. In each case 1 ml. of 0·0002 *N* sulphuric acid containing 20 % alcohol, 0·0008 % methyl red, and 0·00016 % methylene blue, was titrated. The pyrex pipette used for delivering the acid delivered with a coefficient of variation of 0·16 %. The coefficient of variation of the individual titration was 0·85 %. The widest variations in the series were 1·6 and −2·0 % of the mean representing 3·2 and $-4{\cdot}0 \times 10^{-6}$ millimol respectively. The mean error of the burette reading on a single determination was 1·85 mm. This represents $1{\cdot}48 \times 10^{-6}$ of a millimol and corresponds almost exactly to the error described for the Rehberg burette [Rehberg, 1925].

For titrations in atmospheric air it is not possible to diminish this figure appreciably for the above volume by titrating closer to a p_H of 7·0, for any diminution of the buffering power of the water secured thereby is more than counterbalanced by the rapidly increasing buffering power of the carbon dioxide of the air as well as by the instability of the end-point. The carbon dioxide of the air will be in equilibrium with distilled water of approximately p_H 5·6, so that the methyl red indicator has the great advantage that its point of maximum sensitivity lies in the same region.

In a carbon dioxide-free atmosphere and titrating to p_H 7·0 with the above glassware and similar fluids and volumes it is possible to decrease the standard deviation from $1{\cdot}7 \times 10^{-6}$ to $0{\cdot}5 \times 10^{-6}$ millimol KOH, provided we substitute for the indicator mixture one equally sensitive at a p_H of 7·0 and provided also our alkali is perfectly carbon dioxide-free.

The horizontal micro-burette has been found of value in the following procedures.

(1) It may be substituted for the Bang burette over the whole range of micro-titration, being more accurate for the lower ranges (10^{-3} m.eq. or less with acidimetric titration) and of the same accuracy for the higher. It possesses the added advantage of permitting the use of standard solutions ten times more concentrated. The 100 ml. storage bottle will permit the easy filling and delivery of 50 cm. length of the graduated section 250 times without any contact with the carbon dioxide of the atmosphere. The graduated section alone need be cleaned for a smoothly running meniscus.

(2) In the micro-titration of ammonia and urea as described in a previous communication [Conway, 1933] it can be substituted advantageously for the Bang 2 ml. burette. For example in the estimation of urea in 0·2 ml. of blood 1 ml. of 0·1 N sulphuric acid may be placed in the central chamber of the absorbing unit and titrated with 0·025 N carbon dioxide-free alkali. 50 cm. length of alkali will titrate the acid (otherwise a factor is introduced) and the calculation is simply the multiplication by 3 of the cm. of alkali on the graduated tube corresponding to the urea-ammonia. This gives the result in mg. urea per 100 ml. blood.

(3) The burette has been used for the estimation of ammonia in normal blood in conjunction with the absorbing unit described in a previous communication [Conway, 1933] and according to a procedure to be subsequently described.

(4) It has been used for the micro-titration of benzidine sulphate precipitates in centrifuge-tubes in the manner described by Cope [1931] with the Rehberg burette.

(5) It is useful in various micro-thiosulphate titrations permitting the use of comparatively concentrated standard solutions.

(6) It can be used for the very accurate delivery of small volumes of fluid of which a comparatively large quantity is available. It will deliver 0·1 ml. with from one-half to one-third the error of an Ostwald pipette, and more accurately than the ordinary analytical balance can be relied upon to weigh.

(7) It may be used as a very accurate wash-out pipette for very small volumes of blood or serum. In using it for this object water is sucked up in tube F (Fig. 1) to the highest level, the meniscus in the graduated tube being previously adjusted to some point near the end of the graduations. On opening the delivery tap there is now a suction pressure created which may be used to suck in a volume of blood which will be registered by the movement of the meniscus in the graduated tube. The exact volume taken in is thus measured and may then be delivered with an exact volume of wash-out water. It will be seen that this method is very convenient for small volume measurements which need not be exactly represented by tenths of a ml.

Summary.

A simple micro-burette is described which can be easily assembled from pyrex thermometer tubing and graduated tubing. It delivers volumes of the order of 0·1 ml. and may be used for all micro-titrations. It is particularly suitable for titrating quantities of acid of the order 10^{-6} equivalent or less. Its error for delivering 0·1 ml. expressed as a coefficient of variation is 0·12 %. Its error for titrating 1 ml. of 0·0002 N sulphuric acid is $1{\cdot}7 \times 10^{-6}$ expressed as a standard deviation in millimols of alkali.

REFERENCES.

Conway (1933). *Biochem. J.* **27**, 430.
—— and Byrne (1933). *Biochem. J.* **27**, 419.
Cope (1931). *Biochem. J.* **25**, 1183.
Rehberg (1925). *Biochem. J.* **19**, 270.
Stott (1928). Volumetric glassware, p. 32. (Witherby, London.)

XL. VITAMIN A AND CAROTENE.

XI. THE DISTRIBUTION OF VITAMIN A IN THE ORGANS OF THE NORMAL AND HYPERVITAMINOTIC RAT.

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In a previous paper Moore [1931] described the distribution of vitamin A and carotene, as determined by colorimetric methods, in the organs of rats which had received large amounts of carotene as red palm oil or carrot-fat. It was found on the one hand that unchanged carotene persisted throughout the alimentary tract, while on the other a high concentration of vitamin A accumulated in the liver. Outside the liver only small amounts of vitamin were detected. Thus the concentration in the storage fat was only about 1/100,000 of that found in the liver-fat, while in most other tissues, which of course contained only small percentages of fat, the amounts of vitamin present were too small to be detected by colorimetric means. In agreement with previous biological experiments by Sherman and Boynton [1925] however and with the subsequent work of Simmonet and Busson [1932] the kidney and lungs gave faintly positive reactions in some cases. It was concluded that the liver must not only be able to store vitamin A to a much greater extent than any other organ, but must also in a complementary sense play a large part in keeping the concentration of vitamin A throughout the rest of the body within prescribed limits. This question was discussed further in the case of the cow [Moore, 1932, 1].

The experiments described below were carried out as an extension of the previous work in order to keep pace with recent developments in our knowledge of the physiological behaviour and significance of vitamin A as studied from other angles. In the first place work on the vitamin A of human livers obtained at autopsy has shown that chronic nephritis is one of the most noteworthy of those diseases which are characterised by the occurrence of a high proportion of cases having subnormal vitamin A reserves [Wolff, 1932; Moore, 1932, 2], while the same tendency, although to a much less well marked extent, is noticed in bronchitis and pneumonia [Moore, 1932, 2; Fox, 1933]. It is therefore of interest to decide whether measurable amounts of vitamin A are normally present in the kidneys and lungs, and how far these organs are to be differentiated from others in this respect. Secondly it seemed desirable to extend the previous work to include rats which had received large amounts of vitamin A in place of carotene. Finally the ready availability of comparatively pure preparations of vitamin A has led to the confirmation by many workers of Takahashi's original finding that excess of vitamin A has a toxic action [Takahashi *et al.*, 1925; Harris and Moore, 1928; 1929; Drigalski, 1933; Moll *et al.*, 1933; Bomskov and Seemann, 1933;

Collazo and Rodriguez, 1933]. Knowledge of the distribution of vitamin A in the organs of rats suffering from hypervitaminosis must obviously be of interest as a step towards the elucidation of the cause of the toxic action.

Experimental.

Vitamin A was assayed colorimetrically, using the alkali digestion technique [Davies, 1933], in the organs of albino rats which had been given varying amounts of carotene or vitamin A for varying periods. In the case of small organs, or organs containing small amounts of vitamin, the fat obtained after digesting the tissues was made up in 1 or 2 ml. of chloroform instead of in the 5 ml. usually employed in routine work.

The presence of vitamin A in the kidneys of rats having moderate liver reserves.

Five rats which had been used in vitamin A tests, and which presumably possessed negligible vitamin A reserves at the end of the test period, were given for several weeks a diet containing carotene as 15 % of red palm oil, with the addition of carrots in some cases. Vitamin A was assayed in the liver, kidneys, lungs and (in 3 cases) the intraperitoneal fat. The results are given in Table I.

Table I. *Rats having moderate liver reserves.*

	Rat 1 ♂		Rat 2 ♂		Rat 3 ♂		Rat 4 ♂		Rat 5 ♂	
	Total B.U.	B.U. per g.	Total B.U.	B.U. per g.	Total B.U.	B.U. per g.	Total B.U.	B.U. per g.	Total B.U.	B.U. per g.
Liver	900	90	1200	150	1500	150	1000	100	500	50
Kidneys	10	5	6	3	4	3	2	1	8	3
Lungs	6	3	>3	>1·5	0	0	0	0	0	0
Intraperitoneal fat	—	—	—	—	—	0	—	0	—	2

It will be seen that the dietary treatment resulted in the accumulation of moderate vitamin A reserves in the liver[1]. Under these circumstances small amounts of the vitamin were detected in the kidneys in all cases. Only in two out of five was a positive reaction given by the lungs, and in one case out of three by the intraperitoneal fat.

The distribution of vitamin A in the organs of rats given large, but non-toxic, doses of vitamin A concentrate.

Three adult rats were given about 3·5 mg. of B.D.H. vitamin A distillate (3000 B.U. per mg.) each daily for 38 days. They were anaesthetised and killed by bleeding. In order to facilitate the detection of vitamin A in tissues expected to contain only small amounts, the various organs from all three rats were combined after dissection. Results are shown in Table II.

In these rats the liver reserve was extremely high, being 160 times greater than the normal human reserve. The concentration in the kidneys was about 10 times greater than in the previous experiment, but even greater concentrations were found in the lungs and suprarenals, in both of which values were observed

[1] For purposes of comparison it may be mentioned that the "normal" human liver reserve in this country, taken as the median value over a wide range of cases dying by accident, is about 250 B.U. per g. A similar value was obtained in the case of a single wild rat shot in the vicinity of this laboratory.

Table II. *Rats given large, but non-toxic, amounts of vitamin A concentrate.*

Combined tissues from 3 adult ♀ rats, Nos. 6, 7, 8.

	Total B.U. per rat	B.U. per g.
Liver	300,000	40,000
Kidneys	70	50
Lungs	500	450
Suprarenals	100	2,500
Heart	1	1
Spleen	1	2
Pancreas	15	25
Thymus	4	12
Brain	0·5	0·3
Muscles	—	0·5
Blood	—	2
Intraperitoneal fat	—	80

which would be considered quite high for the liver in normally fed rats. In the brain, heart, spleen, muscles and blood only small traces of vitamin were detected, but slightly larger amounts seemed to be present in the pancreas, thymus and intraperitoneal fat. The value in the case of the pancreas may have been made unduly high through the inadvertent inclusion of fat deposits.

To obtain further evidence on the points of interest raised in this experiment further assays were carried out on the liver, kidneys, lungs, pancreas and suprarenals of four adult rats which had received large amounts of vitamin A concentrate. These animals differed from those just described in so far that the feeding of concentrate was discontinued some weeks before killing, an ordinary stock diet of bread and milk, cereals and vegetables being given in the meantime. The organs from each rat were kept separate. Results are given in Table III.

Table III. *Rats given large, but non-toxic, doses of vitamin A concentrate. Concentrate feeding discontinued some time before killing.*

	Rat 9 ♀		Rat 10 ♂		Rat 11 ♀		Rat 12 ♂	
	Total B.U.	B.U. per g.	Total B.U.	B.U. per g.	Total B.U.	B.U. per g.	Total B.U.	B.U. per g.
Liver	75,000	8,000	150,000	15,000	125,000	15,000	125,000	10,000
Kidneys	10	5	10	5	15	10	5	2
Lungs	250	150	60	35	75	50	125	60
Pancreas	0	0	5	5	0	0	0	0
Suprarenals	0	0	2·5	25	5	70	1·5	15

In most cases the concentration of vitamin A in the liver was about half that observed in the preceding experiment. The concentration in the kidneys was remarkably low, being little in excess of that found in the case of the rats having moderate reserves. In the lungs the concentration was again greater than in the kidneys but was much lower than the value found in the previous experiment. The amounts of vitamin present in the suprarenals were much smaller than in the preceding experiment, while in the pancreas a negative result was obtained in three rats, a trace being found in the case of one animal.

The distribution of vitamin A in the organs of rats given toxic doses of vitamin A.

Two young albino rats were given the following diet. "Light white casein" 20 %; rice starch 35 %; coconut oil 40 %; salt mixture 5 %; dried yeast 10 % (additional); radiostol (vitamin D) 1 drop per week. Vitamin A concentrate was given dissolved in the coconut oil at such a concentration that 40 mg. were

included in every 10 g. of diet. To avoid undue loss of the vitamin before ingestion by the rat the solution of the vitamin in the oil was stored separately and admixed with the remaining components of the diet daily immediately before feeding. The history of the rats was as follows.

Rat 13 ♂. Initial weight 95 g. Declined in weight from commencement of the experiment with the development of a hunched attitude, loss of hair round the mouth[1] and haemorrhagic rhinitis. Died after 12 days, final weight 60 g. Average daily food consumption 3·1 g.; consumption of concentrate 12·5 mg. per day. Total concentrate ingested, 150 mg. *Post mortem* examination revealed extreme emaciation and severe lung disease. Parts of the lungs were red, wet and soft, other parts were filled with putty-like hard nodules which were present in all sizes up to about 7 mm. in diameter.

Rat 14 ♀. Initial weight 81 g. Declined in weight from commencement with superficial appearances similar to those observed in the previous rat. Died after 33 days, the final weight being 55 g. Average daily consumption of food 3·6 g., of concentrate 14·3 mg. Total concentrate ingested 471 mg. At autopsy the lungs appeared slightly reddened but did not exhibit the gross lesions observed in the preceding rat. The immediate cause of death appeared to be extreme emaciation. The most striking feature, however, was the extreme softness of the bones. X-ray examination revealed that several of the larger leg bones had sustained spontaneous fractures, the broken ends of the bones being healed together in irregular shapes with the formation of calluses.

Table IV. *Rats given toxic amounts of vitamin A concentrate.*

	Rat 13 ♂		Rat 14 ♀	
	Total B.U.	B.U. per g.	Total B.U.	B.U. per g.
Liver	50,000	15,000	200,000	60,000
Kidneys	40	60	500	600
Lungs	2,500	1,250	1,000	1,000
Suprarenals	0	0	2·5	50
Heart	60	100	15	25
Spleen	0	0	2·5	15
Pancreas	0	0	1	10
Brain	25	15	20	15
Eyes	0	0	0	0
Testicles	0	0	—	—
Muscles	—	0	—	25
Bone	—	0	—	2
Total B.U. ingested	450,000	—	1,400,000	—
Found in faeces	1,500	—	6,400	—
Found in urine	0	—	0	—

The results of vitamin A assays carried out on the organs of these rats are given in Table IV. In the case of the rat dying after 12 days the liver reserve was considerably below the values obtained in some other cases. The lungs contained a very high concentration of vitamin, which in view of the diseased and unduly weighty condition is best judged in this case from the total vitamin present rather than the value expressed per g. of tissue. Considerable amounts of vitamin were present in the kidney and heart. A small amount was found in the brain. Other organs gave negative results.

[1] The loss of hair round the mouth would appear to be due to the irritant action of traces of diet remaining on the skin after feeding. It is noteworthy, however, that the concentrate exerts no immediate irritant action. Our solution of the vitamin A concentrate in coconut oil could be eaten by ourselves in small amounts without irritation or discomfort.

In the case of the rat dying after 33 days the concentration of vitamin A in the liver was four times greater than in the preceding rat. The total amount of vitamin present in the lungs was less, but in this case a high value was found for the kidneys. Significant amounts of vitamin were also found in all organs except the eyes[1].

In order to determine the efficiency of the absorption of vitamin A in these rats the faeces and urine were collected daily and stored in 5 % KOH until the end of the experiment, when vitamin A assays were carried out on aliquot portions by the usual technique. In each case the urine gave a negative result. Positive results were found for the faeces, but the amounts present were insignificantly small when compared with the difference between the amount of vitamin ingested and that found in the body. In each case only about $\frac{1}{7}$ of the ingested vitamin could be accounted for, the remainder presumably being lost by oxidation at some stage as yet unknown.

The distribution of vitamin A in the organs of rats given large doses of carotene.

Two albino rats from the same litter as those used in the preceding experiment were given the same diet except for the substitution of carotene (B.D.H.) in place of vitamin A. Owing to the limited solubility of the pigment it was found impossible to incorporate more than 10 mg. per 10 g. of diet (*i.e.* $\frac{1}{4}$ of the concentration of vitamin A in the preceding experiment), but since the food consumption was much greater the actual daily consumption of carotene was about half that of vitamin A. The animals grew rapidly and when killed after 34 days were found at autopsy to be perfectly normal. The storage fat was plentiful and quite colourless. Particulars were as follows.

Rat 15 ♂. Initial weight 95 g. Final weight 156 g. Average daily food consumption 8·3 g.; carotene consumption 8·3 mg. daily; total carotene ingested 281 mg.

Rat 16 ♀. Initial weight 83 g. Final weight 129 g. Average daily food consumption 7·9 g.; carotene consumption 7·9 mg. daily; total carotene consumption 268 mg. The distribution of vitamin A in the organs of these rats is shown in Table V.

Table V. *Rats given large amounts of carotene.*

	Rat 15 ♂		Rat 16 ♀	
	Total B.U.	B.U. per g.	Total B.U.	B.U. per g.
Liver	12,500	1,250	12,500	1,700
Kidneys	5	4	5	3
Lungs	30	30	20	12
Suprarenals	0	0	0	0
Heart	0	0	0	0
Spleen	1·5	3	0	0
Pancreas	2·5	10	0	0
Brain	0	0	0	0
Eyes	0	0	0	0
Testicles	0	0	—	—
Muscles	—	0	—	0
Bone	—	0	—	0
Intraperitoneal fat	—	5	—	2
Y.U. carotene ingested	560,000	—	530,000	—
Found in faeces	55,000	—	25,000	—

[1] In view of the fact that the retina has been reported to contain considerable amounts of vitamin A [Smith *et al.*, 1931; Wald, 1933] this negative result should not be taken to imply that the vitamin was entirely absent, but it is evident that the concentration of the vitamin, if present, was not sufficiently high to compensate for the small amounts of tissue available for assay.

The vitamin A reserves found in the liver would represent high values for ordinary conditions of nutrition but are some 30 times lower than the maximum value obtained by giving vitamin A. In the kidney the concentration of vitamin was no greater than in the rats having only moderate liver reserves, but in the lungs the amounts present were of a slightly higher order. In each case a faintly positive reaction was given by the intraperitoneal fat, while in rat 15 traces of chromogen were also detected in the spleen and pancreas. In the case of the spleen the yellow/blue ratio indicated that the reaction was due to carotene.

In order to determine the efficiency of absorption of the carotene the faeces and urine were examined as in the preceding experiments, the amounts found representing about 10 % and 5 % of the ingested pigment in rats 15 and 16 respectively. Since the amount of vitamin A present in the body must in each case have represented only some 3–6 mg. of carotene, or about 1–2 % of the ingested carotene, it is obvious that the utilisation of carotene at such high levels must be very inefficient, and that the greater part of the pigment has not been accounted for.

Discussion.

The distribution of vitamin A in the organs of the rat.

The above experiments indicate that small amounts of vitamin A (*ca.* 5 B.U. per g.) are consistently present in the kidneys even when the liver reserves are of a moderate order (*ca.* 100 B.U. per g.). Traces of the vitamin are sometimes present in the lungs under these conditions.

When the liver reserve is raised by the inclusion in the diet of large amounts of carotene or vitamin A the order of the kidneys and the lungs is reversed, since the lungs now invariably show a greater concentration of vitamin A. As far as can be judged both from the present experiments and from previous work [Moore, 1931] carotene feeding does not result in the accumulation of vitamin A in high concentration in any organ except the liver, the highest value observed being 30 B.U. per g. for the lungs of rats Nos. 15 and 16 in the present work. The feeding of vitamin A concentrate on the other hand may raise the concentration in the kidneys and lungs to values that would be high even for the liver in the case of animals receiving ordinary diets. At the same time appreciable amounts of vitamin may appear throughout the whole body. In the case of the suprarenal glands very inconsistent results have been obtained. For rats 6, 7 and 8 a very high average value (2500 B.U. per g.) was observed, but in other experiments the results were much lower and in some cases negative. It may be noted that in view of the small size of these organs the actual amount of vitamin involved was relatively small.

Hypervitaminosis A.

The production of toxic symptoms in the present work may be considered to provide further evidence as to the reality of hypervitaminosis A in so far as the preparation used was a distillate, as opposed to the non-distilled concentrates used, as far as we are aware, by other workers in this field. Drigalski [1933] has already shown that the toxic power of the concentrates is destroyed with the vitamin on ultra-violet irradiation, it is now evident that the vitamin potency and toxic power are stable to distillation and are not separated by this means. Although only two rats were used in the present work we were fortunate in observing almost all the symptoms described by previous workers. The lung lesions observed in rat 13 exactly resembled those described in several cases by Takahashi *et al.* [1925]. It is perhaps noteworthy that these lesions should occur

in the organ in which the greatest concentration of vitamin A outside the liver was located. In rat 14 the bone lesions were exactly similar to those described by Bomskov and Seemann [1933] and also Collazo and Rodriguez [1933], our own observations representing the third independent discovery of the phenomenon.

Speculation as to the mechanism of the production of hypervitaminosis A would be premature at the present stage, but two clearly established points may be worth mention. In the first place it is obvious that the liver possesses a very high toleration for the vitamin, since concentrations of the order observed in the hypervitaminotic rats (Nos. 13 and 14) were also observed in healthy animals (Nos. 6, 7 and 8). At the maximum level observed the vitamin must have constituted about 5 % of the dry weight of the liver and have represented a supply sufficient to last the rat for about 200 years if economically used. Secondly it is plain that in the case of rat 13 death through hypervitaminosis intervened at a point when the liver still contained less than half the maximum observed concentration of vitamin. This finding shows that death may occur while there is still room for a considerably greater accumulation of vitamin A in the liver and suggests that the production of hypervitaminosis does not depend on the absolute amount of vitamin A present in the organism but on the ingestion of the vitamin at a greater rate than that at which it can be stored by the liver or eliminated by the organism.

The absence of toxic symptoms after feeding excess of carotene.

The absence of any symptoms of hypervitaminosis in the rats (Nos. 15 and 16) given about 8 mg. of carotene daily suggests that it is virtually impossible to induce hypervitaminosis A by carotene feeding. This conclusion has already been reached by Kuhn and Brockmann [1933], but since these workers administered carotene at the rate of only about 1 mg. per day, which is little greater than the dosage of 0·75 mg. used by Moore [1930] in earlier experiments, it is obvious that further evidence on this point was desirable.

It is still possible, of course, that large masses of solid carotene, as opposed to solutions of carotene in oil, might possess toxic action. Even if this proved to be the case, however, the phenomenon observed would almost certainly be quite different from the hypervitaminosis induced by giving vitamin A concentrate, since a comparison of the vitamin A contents of the livers of rats 13 and 14, which were given large amounts of vitamin A concentrate, with those of rats 15 and 16, which were given large amounts of carotene, indicates that the rate at which the liver reserve can be raised by the conversion of carotene (50 B.U. per g. per day) is much slower than the rate of increase obtained by giving vitamin A (1200–1700 B.U. per g. per day). The superior rate and efficiency of absorption of vitamin A suggest that in intensive vitamin A therapy preformed vitamin A should be more efficacious than carotene. At levels approaching the minimum dose, as previously shown [Moore, 1933], vitamin A and carotene appear to be equally efficiently utilised.

Summary.

1. Vitamin A was assayed by the antimony trichloride method in the organs of rats which had received varying amounts of vitamin A or carotene.

2. Even when the liver reserves were quite low (100 B.U. per g.) small amounts of vitamin A (5 B.U. per g.) were found in the kidneys and sometimes in the lungs.

3. When large, but non-toxic, doses (3·5 mg. daily) of vitamin A distillate were given to adult rats (*a*) the concentration of vitamin in the liver rose to

extremely high levels (40,000 B.U. per g.), (*b*) the concentration in the lungs was of the order found in the liver under normal conditions of nutrition, (*c*) the concentration in the kidney remained fairly low (50 B.U. per g.), (*d*) the concentration in other organs was extremely low, except that surprisingly high values were observed in the suprarenals (up to 2500 B.U. per g.).

4. Even more massive doses of the distillate (12–14 mg. per day) given to young rats proved toxic, causing emaciation, haemorrhagic rhinitis, loss of hair round the mouth and (*a*) in one rat, dying after 12 days, gross lung lesions, (*b*) in another rat, dying after 33 days, a peculiar softening and hollowing of the bones which had resulted in spontaneous fractures of the legs. The distribution of vitamin A resembled that found in rats given non-toxic doses except that the concentration in the lungs was even higher (1250 B.U. per g.), while in the rat dying after 33 days a high kidney value (625 B.U. per g.) was observed.

5. Attempts to induce hypervitaminosis A by feeding carotene failed. On account of its limited solubility in fats only 8 mg. of the pigment could be given daily. Rats receiving this amount grew rapidly and remained in perfect health. On killing after 34 days the liver reserve was found to be only moderately high (1250 B.U. per g.) pointing to only about 1 % absorption, as opposed to 15 % in the case of vitamin A. Only very small amounts of vitamin were present in the lungs and kidneys, negative results being obtained in all other organs.

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REFERENCES.

Bomskov and Seemann (1933). *Z. ges. exp. Med.* **89**, 771.
Collazo and Rodriguez (1933). *Klin. Woch.* **12**, 1732, 1768.
Davies (1933). *Biochem. J.* **27**, 1770.
Drigalski (1933). *Klin. Woch.* **12**, 308.
Fox (1933). *Lancet*, i, 933.
Harris and Moore (1928). *Biochem. J.* **22**, 1473.
—— —— (1929). *Biochem. J.* **23**, 1120.
Kuhn and Brockmann (1933). *Klin. Woch.* **12**, 972.
Moll, Domagh and Laquer (1933). *Klin. Woch.* **12**, 465.
Moore (1930). *Biochem. J.* **24**, 696.
—— (1931). *Biochem. J.* **25**, 275.
—— (1932, 1). *Biochem. J.* **26**, 1.
—— (1932, 2). *Lancet*, ii, 669.
—— (1933). *Biochem. J.* **27**, 898.
Sherman and Boynton (1925). *J. Amer. Chem. Soc.* **47**, 1646.
Simmonet and Busson (1932). *Compt. Rend. Soc. Biol.* **109**, 182.
Smith, Yudkin, Kriss and Zimmerman (1931). *J. Biol. Chem.* **92**, *Proc.* xcii.
Takahashi, Nakamiya, Kawakami and Kitasato (1925). *Scient. Papers of Inst. of Chem. and Phys. Res.* (Tokyo), **3**, 81.
Wald (1933). *Nature*, **132**, 316.
Wolff (1932). *Lancet*, ii, 617.

XLI. THE INACTIVATION OF ANIMAL AMYLASE BY PLANT PARALYSERS AND THE PRESENCE OF INACTIVATING SUBSTANCES IN SOLUTIONS OF ANIMAL AMYLASE.

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In the preceding papers [1933] it was shown that in germinated and ungerminated cereals there are natural paralysers of amylase, which we call "sistoamylase substances." Buckwheat malt contains many of these paralysers, mostly in the leaves and roots. The sistoamylase adsorbs the plant amylase either in a state of solution or at the moment of being dissolved and so inactivates it.

Moreover we have found other substances, which counteract the sistoamylase and have named these substances "eleutoamylases." Any eleutoamylase added to the system sistoamylase-amylase prevents inactivation by the paralyser, or if the amylase has been already inactivated by the sistoamylase, the whole or a greater part of the inactivated amylase is put into action by the eleutoamylase. The most active eleutoamylases recognised by us are products of decomposition of protein found in peptone.

In the present paper the same problem is discussed in relation to animal amylase. Before anything else is suggested the following questions should be answered:

(1) Is plant sistoamylase able also to inactivate animal amylase?

(2) Does the system composed of plant sistoamylase and animal amylase react like the similar system containing plant amylase, the amylase of which can be reactivated by adding a suitable eleutoamylase?

(3) Do there co-exist in the animal organism besides amylases sisto-substances characterised by properties similar to those of the plant sistoamylases?

Experimental.

The experiments were conducted on human saliva, collected on the day of starting experiments, and on Merck's pancreatic amylase. The saliva was diluted in the proportion 5:100, well mixed during 5 mins. and filtered. The solutions of pancreatic amylase were prepared by dissolving 0·25 g. of pancreatic amylase in 100 ml. of water at 20°, shaken during 5 mins. and then filtered. The leaves and roots of buckwheat-malt, germinated within 15 days at 15°, were taken as material containing the plant paralyser, the sistoamylase substance. This material was used in two different manners: well crushed undried, and dried in the apparatus of Faust-Heim at 35° and then reduced to powder. "Peptone siccum sine sale" (Merck) served as eleutoamylase. The p_H of Merck's soluble starch solution was adjusted by additions of phosphate buffers to 6·7, determined

potentiometrically. The amylolytic activities of the solutions of amylase were measured by their liquefying, dextrinising and saccharifying powers.

The liquefying power of saliva was determined with native starch according to the method of Lintner-Sollied [1902], but at 40° instead of 65°. The liquefying power of pancreatic amylase was established by the method of Pollak [1903] at 40°.

The dextrinising power. For pancreatic amylase a 2·5 % and for salivary amylase a 1 % solution of Merck's soluble starch was prepared, by addition of starch paste to boiling water, boiling the solution for a further 3 mins., and, after cooling it, adjusting the p_H. 50 ml. of this solution were warmed for 30 min. at 40°, afterwards 5 ml. of the enzyme solution to be tested were introduced. At intervals of 4 mins., 1 ml. of this system was taken and added to 1 ml. of iodine solution (0·8 g. iodine in 1 litre H_2O) and the colour obtained was observed. The dextrinising activity was measured by the time required for obtaining the following colours: violet, brownish red, light yellow.

The saccharifying power was investigated with the same solutions of soluble starch also at 40°; 5 ml. of the enzyme solution were added to 50 ml. of the solution of soluble starch and treated as before. After 15 mins. 10 ml. of $N/10$ NaOH were added, the mixture was cooled, and reducing sugars were determined in 30 ml. by the method of Willstätter and Schudel [1918]. The saccharifying power is expressed by the number of ml. of $N/20$ iodine reduced by 30 ml. of the liquid tested. The amount of iodine reduced by the control test was deducted.

I. Salivary amylase.

(a) *Inactivation of salivary amylase by plant paralyser.*

In order to ascertain whether the plant paralyser also inactivates the amylase of saliva and in what degree, plant paralyser was taken from two sources: dried leaves and rootlets of buckwheat-malt (1) obtained a year ago and (2) obtained shortly before our experiments. The mixtures used were prepared by addition of the quantities of sistoamylase substance shown in Table I to 40 ml. of 5 % saliva. In each case (except No. 1) the solution was shaken mechanically for 8 mins. and then filtered, and the amylolytic activity of the filtrate was investigated (Table I).

Table I.

No. of solution	Additions g.	Saccharifying power ml. $N/20$ I	Dextrinising power: Violet was obtained with iodine in mins.	Dextrinising power: Brownish red was obtained with iodine in mins.	Dextrinising power: Light yellow was obtained with iodine in mins.	Liquefying power Lintner-Sollied method 1 g. starch is liquefied by 5 % saliva, ml.
1	None	1·8	20	40	220	1·8
2	0·1 sistoamylase 1	0·1	110	180	Not obtained in 240 mins.	2·4
3	0·3 ,,	0·1	Without change after 240 mins.			Not liquefied by 4 ml.
4	0·5 ,,	0·1	,,			,,
5	0·7 ,,	0·1	,,			,,
6	0·1 sistoamylase 2	0·1	190	Not obtained after 240 mins.		,,
7	0·3 ,,	0·1	Without change after 240 mins.			,,
8	0·5 ,,	0·1	,,			,,
9	0·7 ,,	0·1	,,			,,

These experiments demonstrate that a plant paralyser also inactivates salivary amylase: 0·3 g. of sistoamylase substance is sufficient to inactivate completely 40 ml. of 5 % saliva in 8 mins. The dried sistoamylase substance obtained shortly before the experiment possesses the inactivating power in a higher degree than that obtained a year ago.

(b) The inactivation of salivary amylase by sistoamylase substance in presence of peptone.

(1) *Peptone an antisistoamylase substance to salivary amylase.* The next point which we investigated was the inactivation of salivary amylase by a dried paralyser in presence of peptone. Peptone was added simultaneously with the sistoamylase to the solution of salivary amylase. The solutions used were made up by addition of the substances shown in Table II to 40 ml. of 5 % saliva. In each case the solution was mechanically shaken for 8 mins. and then filtered, and the amylolytic action of the filtrate was investigated (Table II).

Table II.

No. of solution	Additions: Peptone g.	Additions: Sistoamylase substance g.	Saccharifying power ml. $N/20$ I	Dextrinising power: Violet	Dextrinising power: Brownish red	Dextrinising power: Light yellow	Liquefying power Lintner-Sollied method 1 g. starch is liquefied by 5 % saliva, ml.
				was obtained with iodine in mins.			
1	None	None	1·6	60	90	240	1·8
2	0·5	,,	4·5	10	19	84	1·2
3	1·0	,,	3·9	10	16	66	0·9
4	2·0	,,	3·5	9	15	54	0·9
5	None	0·3 Prep. 1	0·1	Without change after 4 hours			Not liquefied by 4 ml.
6	,,	0·3 ,, 2	0·1	,,	,,	,,	,,
7	0·5	0·3 ,, 1	2·0	15	30	130	1·2
8	1·0	0·3 ,, 1	3·8	14	21	84	1·0
9	2·0	0·3 ,, 1	3·1	10	17	60	1·0
10	0·5	0·3 ,, 2	2·6	18	32	132	2·1
11	1·0	0·3 ,, 2	3·8	12	21	84	1·0
12	2·0	0·3 ,, 2	3·0	11	18	58	1·0

The experiments demonstrate that treatment with peptone greatly increases the amylolytic activity of salivary amylase. This fact can be explained on the ground that in saliva only a small quantity of the amylase is active. The greater part is inactivated, and its activity can be restored by addition of peptone. Evidently in the diluted saliva there must be an inactivating substance with the properties of our sistoamylase. It is shown later that the plant paralyser does not inactivate salivary amylase in the presence of peptone. These results are in agreement with those obtained for plant amylase. It is interesting that peptone has a different effect on the liquefying, dextrinising and saccharifying powers of salivary amylase.

(2) *Peptone an eleutoamylase substance to salivary amylase.* In order to ascertain whether eleutoamylase restores the activity of salivary amylase which has been inactivated by the plant paralyser (dried or undried) the following solutions were used.

No. of solution															
1	40 ml. 5 % saliva	...	...	...	...	...	...	...	...	...	...	...	...	...	filtered
2	,,	+0·3 g. dried sistoamylase.				(1)	Shaken	10	mins.	and then	+2 g. peptone,	eluted	15	mins. then	filtered
3	,,	,,		,,		,,		10	,,		,,		30	,,	,,
4	,,	,,		,,		,,		10	,,		,,		60	,,	,,
5	,,	,,		,,	(2)	,,		10	,,		,,		15	,,	,,
6	,,	,,		,,		,,		10	,,		,,		30	,,	,,
7	,,	,,		,,		,,		10	,,		,,		60	,,	,,
8	,,	+1 g. undried		,,		,,		8	...	...	...	...	...	...	then ,,
9	,,	,,		,,		,,		8	,,		+2 g. peptone,	eluted	10	mins. then	,,
10	,,	,,		,,		,,		15	,,		,,		10	,,	,,
11	,,	,,		,,		,,		30	,,		,,		10	,,	,,
12	,,	,,		,,		,,		60	,,		,,		10	,,	,,
13	,,	,,		,,		,,		8	,,		,,		30	,,	,,
14	,,	,,		,,		,,		15	,,		,,		30	,,	,,
15	,,	...	...	...	...	...	...	...	...	...	+2 g. peptone,	shaken	8	,,	,,

The amylolytic action of the filtrates was then investigated.

Table III.

No. of solution	Saccharifying power ml. $N/20$ I	Dextrinising power: Violet was obtained with iodine in mins.	Dextrinising power: Brownish red was obtained with iodine in mins.	Dextrinising power: Light yellow was obtained with iodine in mins.	Liquefying power Lintner-Sollied method 1 g. of starch is liquefied by 5 % saliva, ml.
1	1·6	60	90	240	1·8
2	0·1	Without change after 4 hours			Not liquefied by 4 ml.
3	0·1		,,		,,
4	0·1		,,		,,
5	0·1		,,		,,
6	0·1		,,		,,
7	0·1		,,		,,
8	0·0		,,		,,
9	1·7	24	42	120	1·2
10	1·0	26	54	Not obtained	1·3
11	0·6	48	90	,,	1·4
12	0·6	90	174	,,	1·7
13	1·7	24	40	124	0·9
14	1·1	34	48	218	1·1
15	5·8	9	12	42	0·3

The dried plant paralyser inactivates the amylolytic action of salivary amylase so strongly that even prolonged contact of peptone with adsorbed amylase does not lead to an elution of the ferment, and such a system does not exhibit amylolytic activity. After treatment with undried plant paralyser on the other hand peptone reactivates the salivary amylase. These results are in agreement with those obtained on plant amylase.

It remains to be established whether the addition of peptone to the system undried sistoamylase + salivary amylase, the amylolytic action of which is nearly nil, actually leads to elution of amylase or whether a small part of the amylase undergoes no adsorption and gives the corresponding intensive amylolytic action with peptone. To this end, the following solutions were prepared. To 40 ml. of 5 % saliva was added 1 g. undried sistoamylase, the mixture shaken for a definite time and filtered during 6 mins. The nature of the sistoamylase added and the times of shaking are shown below.

No. of solution	Duration of malting in prep. of sistoamylase days	Duration of shaking mins.
1	11	8
2	11	12
3	11	27
4	11	57
5	25	8
6	25	27
7	25	57

Two series of experiments were made by establishing the dextrinising and saccharifying powers of each of the above solutions after filtration. The first series (*a*) without any addition, the next (*b*) with the addition of 0·2 g. peptone to 50 ml. of 1 % soluble starch solution. If peptone does not elute but only increases the amylolytic activity of the inactivated solutions, the numbers of series "*b*" must agree with the results in solutions 9–14 in Table III.

In Table IV we see that the young undried sistoamylase substance produced in 11 days' malting adsorbs the salivary amylase quantitatively. But the

Table IV.

No. of solution	Saccharifying power ml. $N/20$ I	Dextrinising power: Violet	Brownish red	Light yellow (was obtained with iodine in mins.)	Liquefying power Lintner-Sollied method 1 g. of starch is liquefied by 5 % saliva, ml.
1 *a*	0·0	Without change after 4 hours			Not liquefied by 4 ml.
1 *b*	0·0	,,			,,
2 *a*	0·0	,,			,,
2 *b*	0·0	,,			,,
3 *a*	0·0	,,			,,
3 *b*	0·0	,,			,,
4 *a*	0·0	,,			,,
4 *b*	0·0	,,			,,
5 *a*	0·0	,,			,,
5 *b*	0·1	,,			,,
6 *a*	0·0	,,			,,
6 *b*	0·1	120	180	Not obtained	,,
7 *a*	0·1	Without change after 4 hours			,,
7 *b*	0·1	110	160	Not obtained	,,

undried sistoamylase substance produced in 25 days also adsorbs the amylase at first very strongly; however, shaking the system for a long time has the effect that a small quantity of the sistoamylase with adsorbed amylase passes into solution. This dissolved system does not itself show any amylolytic action, but when peptone is added, as in the experiment with the solutions of starch of series "*b*," this system begins to act, the action of the sistoamylase being eliminated.

II. Pancreatic amylase.

(*a*) *Inactivation of pancreatic amylase by plant paralyser.*

To ascertain whether the plant sistoamylase is able to inactivate pancreatic amylase, the following experiments were made. Solutions of 0·25 g. pancreatic amylase in 100 ml. of water were shaken mechanically for 5 mins. and then various quantities of sistoamylase were added, the solutions shaken for 8 mins. and filtered. The amylolytic power of the filtrates was then examined (Table V).

Table V summarises the results.

Table V.

No. of solution	Additions g.	Saccharifying power ml. $N/20$ I	Dextrinising power: Violet	Brownish red	Light yellow (was obtained with iodine in mins.)	Liquefying power Pollak method 0·3 g. starch is liquefied by 5 % saliva, ml.
1	None	3·9	80	150	Not obtained	0·5
2	0·1 sistoamylase 1	1·7	150	240	,,	0·7
3	0·3 ,,	0·2	Without change after 4 hours			Not liquefied by 3 ml.
4	0·5 ,,	0·1	,,			,,
5	0·7 ,,	0·1	,,			,,
6	0·1 sistoamylase 2	0·7	Nearly without change after 4 hours			1·0
7	0·3 ,,	0·1	,,			Not liquefied by 3 ml.
8	0·5 ,,	0·1	Without change after 4 hours			,,
9	0·7 ,,	0·0	,,			,,

These results show that the plant sistoamylase also inactivates pancreatic amylase. 0·3 g. of the sistoamylase preparations inactivates 100 ml. of 0·25 % pancreatic amylase in 8 mins.

(b) The inactivation of pancreatic amylase by sistoamylase in presence of peptone.

(1) *Peptone an antisistoamylase substance to pancreatic amylase.* The effect of peptone on the sistoamylase-pancreatic amylase system was investigated in a similar manner, filtered solutions of 0·25 g. pancreatic amylase in 100 ml. water being used and shaken for 8 mins. after the additions had been made.

Table VI.

No. of solution	Additions	Saccharifying power ml. N/20 I	Dextrinising power: Violet (was obtained with iodine in mins.)	Dextrinising power: Brownish red (was obtained with iodine in mins.)	Dextrinising power: Light yellow (was obtained with iodine in mins.)	Liquefying power Pollak method 0·3 g. starch is liquefied by 5 % saliva, ml.
1	None	3·9	98	156	Not obtained	0·4
2	0·3 g. dried sistoamylase	0·1	Without change after 4 hours			Not liquefied by 3 ml.
3	„ +2 g. peptone	16·6	20	30	85	0·2
4	2 g. peptone	16·8	18	28	78	0·1

This table shows that only a very small quantity of free amylase is present in the solution of pancreatic amylase. Besides this, there is about four times as much amylase inactivated by a specific paralyser. The latter can be put out of action by peptone. We see further that the peptone acts also as anti-substance to added plant paralyser, just as in the case of the salivary amylase.

(2) *Peptone an eleutoamylase substance to pancreatic amylase.* In order to prove that peptone has the capacity of eluting the pancreatic amylase from the system pancreatic amylase+plant paralyser, the amylolytic activity of the following solutions was examined:

No. of solution																		
1	0·25 g. of pancreatic amylase+100 ml. of water 5 mins. mechanically shaken and	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	filtered	
2	As above	Afterwards	+0·3 g. dried sistoamylase.				Shaken	8	mins. and then	+2 g. of peptone,			eluted	10	mins. and	„		
3	„	„	+0·3	„	„	„	8	„	„	„				30	„	„		
4	„	„	+0·3	„	„	„	8	„	„	„				60	„	„		
5	„	„	+1·0 g. undried		„	„	8	„	...	...	...	...	...	...	and	„		
6	„	„	„	„	„	„	8	„	+ 2g. of peptone,				eluted	10	mins. and	„		
7	„	„	„	„		„	15	„	„					10	„	„		
8	„	„	„	„	„	„	30	„	„					10	„	„		
9	„	„	„	„	„	„	60	„	„					10	„	„		
10	„	„	„	„	„	„	8	„	„					30	„	„		
11	„	„	„	„	„	„	15	„	„					30	„	„		
12	„	...	...	...	...	...	...	...	...	...	+2 g. of peptone,			...	...	„		

These experiments (Table VII) show that dried sistoamylase adsorbs the pancreatic amylase much more strongly than undried. We see also that the adsorption of the pancreatic amylase is the more complete, the longer the undried sistoamylase acts, so that it becomes more and more difficult to elute the amylase.

Table VII.

No. of solution	Saccharifying power ml. N/20 I	Dextrinising power: Violet (was obtained with iodine in mins.)	Dextrinising power: Brownish red (was obtained with iodine in mins.)	Dextrinising power: Light yellow (was obtained with iodine in mins.)	Liquefying power Pollak method 0·3 g. starch is liquefied by 5 % saliva, ml.
1	3·9	98	156	Not obtained	0·4
2	0·1	Nearly without change after 4 hours			Not liquefied by 3 ml.
3	0·4	210	Not obtained		2·8
4	0·7	180	220	Not obtained	2·0
5	0·1	Nearly without change after 4 hours			Not liquefied by 3 ml.
6	11·1	28	41	106	0·1
7	9·0	35	48	135	0·1
8	7·0	45	60	192	0·15
9	6·3	52	73	216	0·2
10	12·0	24	46	94	0·1
11	8·6	36	53	192	0·15
12	16·8	18	28	78	0·1

Discussion.

Our researches on animal amylase give us not only an explanation of its relations to the natural plant paralyser, our sistoamylase substance, but also explain the manner in which different systems of animal amylase act under the influence of eleuto-substances.

We have ascertained that animal amylase, similarly to vegetable amylase, shows, depending on its origin and conditions of action, different mutual ratios of the three functions of amylolytic activity, the liquefying, dextrinising and saccharifying powers.

We see further that animal amylase under the influence of a plant paralyser is inactivated. This inactivation is of different grade depending on the sistoamylase substance, on the time of its activity and on the origin of the amylase. It is to be noticed that dried sistoamylase substances have a greater power of adsorption than undried. The degree of adsorption increases with the time of action of the paralyser on the amylase.

The most interesting result of our investigation is that in salivary and pancreatic amylases substances inactivating the amylase are found. The result of this is that only a part of amylase is able to act, *e.g.* in a solution of salivary amylase only about 1/3 and in a solution of pancreatic amylase only about 1/4–1/5 of the total amylase is free. It can be deduced that natural paralysers of amylase, sistoamylase substances, occur along with the amylase in the animal, just as in the vegetable, organism. Peptone acts towards the plant paralyser in the same way as to the paralyser found in the solutions of animal amylase. Peptone added to the system plant sistoamylase + animal amylase prevents permit inactivation of the amylase, and also has the power of reactivating the amylase already inactivated by the sistoamylase substance originally present. The adsorption of animal amylase by an animal sisto-substance seems not to be so strong as the adsorption in the system plant sistoamylase + animal amylase. From the first system, especially in solutions of pancreatic amylase, the amylase is freed with more facility, and its amylolytic activity is restored. From the second system the amylase is freed with more difficulty, and in the system dried plant sistoamylase + animal amylase the amylase is so strongly adsorbed that it can only be eluted with difficulty by adding peptone. It is an interesting fact that peptone does not act on all the functions of the amylase equally. As

shown by our experiments, the saccharifying power is maximum on addition of 0·5 g. of peptone to 40 ml. of 5 % solution of salivary amylase. The dextrinising and liquefying powers show the best activity when 1–2 g. peptone are added to the same quantity of solution.

The question of the paralyser of amylase was treated for the first time by Wolff and Fernbach [1906], who introduced into the literature the term anti-amylase for the agent obtained by subcutaneous immunisation of rabbits to malt amylase. This phenomenon has been the subject of numerous researches by different workers. These investigations show that the anti-amylase acts as an inhibitor only on vegetable amylase. Abderhalden and Wertheimer [1922] not only question its paralysing action, but reject in general the existence of this anti-amylase. Kiselew [1928] distinguishes another kind of inactivation in the adsorption of amylase by vegetable plasma, coagulated by warming. Oparin and Kurssanow [1929] showed that in these cases the result was not due to the specific action of the plasma but to the inhibiting action on amylase caused by the presence of tannin.

The most interesting researches in this direction are those of Lesser [1919; 1921] and of Kerner and Lesser [1919], who proved that amylase of the liver is partly inactivated by adsorption, and that the retardation of the decomposition of glycogen may take place not only *in vivo*, but may also be reproduced experimentally by the addition of some adsorbent to the solution. The development of the details of this question is given by Przyłęcki *et al.* [1927]. Lesser showed, using adrenaline as stimulant, that the amylase of the liver can be freed, so that the active concentration of the ferment increases by elution of the inhibitor. Truszkowski [1928] finds that amylase can be inactivated by adsorption on lipoids to the extent of about 67 % of that present in the solution. The amylolytic action of the inactivated amylase can be restored almost quantitatively by the addition of an appropriate quantity of propyl or butyl alcohol.

The activating influence of peptone and of amino-acids is described by Effront [1900] and by Pozerski [1902]. Oppenheimer's [1927] opinion is that here we have to do with a change of p_H, though it is not excluded that these substances may have a real stimulating influence, as was proved by the researches of Sherman *et al.* [1921; 1922] and Rockwood [1924], who took the p_H into consideration. Oppenheimer supposes that this phenomenon can be explained on the ground that peptone protects the amylase from inactivation by rise of temperature.

According to our experiments the so-called phenomenon of activation of animal amylase is to be explained quite differently. We have shown that the whole of the amylase is not active, but only a portion of it. Very often a large portion of it is inactivated by adsorption by different accompanying substances, which are called by us sistoamylase substances. If peptone is added to a system of sistoamylase-amylase the action of the sistoamylase is excluded, the active concentration of amylase increases by elution, and in this case, we often find in the solution several times more free amylase, which according to its quantity will develop a correspondingly greater activity. The increase of amylolytic activity when different substances are added to the solution of animal amylase is nothing else than an elution of amylase from the system sistoamylase-amylase, in which a part of the amylase was adsorbed by the accompanying adsorbent. We are inclined to believe that various phenomena of the protection of amylase from inactivation, as for instance by moderate use of temperature, are in reality complete or incomplete hindrances of adsorption of the amylase by substances already present or formed during the treatment.

SUMMARY.

Experiments on salivary and pancreatic amylase lead us to the following results.

1. Plant sistoamylase inactivates not only plant but also animal amylase. Dried sistoamylase inactivates amylase more powerfully than undried.

2. In the place of origin of amylases as well as in their solutions natural paralysers, the so-called animal sistoamylases, are also found. The system sistoamylase-amylase is thus formed, in which the amylase is partly inactivated. Usually about 1/3 of the salivary and about 1/5 of the pancreatic amylase is not adsorbed.

3. In the system plant or animal sistoamylase-animal amylase, in which the amylase is inactivated, the amylase can be reactivated by the aid of eleutosubstances of which one of the best known by us is peptone. Certain forms of dried sistoamylase are of such adsorbing power that the amylase cannot be eluted by the use of peptone.

4. We explain the increase of amylolytic action of amylase on the addition of certain substances as an elution of amylase, which in this manner is rendered free to act.

REFERENCES.

Abderhalden and Wertheimer (1922). *Fermentforsch.* **6**, 286.
Chrząszcz and Janicki (1933). *Biochem. Z.* **260**, 354; **263**, 250; **264**, 192; **265**, 260.
Effront (1900). Les diastases. (Paris.)
Kerner and Lesser (1919). *Biochem. Z.* **102**, 284.
Kiselew (1928). *Dnewnik d. Bot. Tagung*, 23.
Lesser (1919). *Biochem. Z.* **102**, 304.
—— (1921). *Biochem. Z.* **119**, 108.
Lintner-Sollied (1902). *Z. ges. Brauw.* **26**, 329.
Oparin and Kurssanow (1929). *Biochem. Z.* **209**, 181.
Oppenheimer (1927). Die Fermente, 701. (Thieme, Leipzig.)
Pozerski (1902). *Malys Jahresber.* 460.
Pollak (1903). *Woch. Brau.* **20**, 595.
Przyłęcki, Niedzwiecka and Majewski (1927). *Biochem. J.* **21**, 1025.
Rockwood (1924). *J. Amer. Chem. Soc.* **46**, 1641.
Shermann and Caldwell (1921). *J. Amer. Chem. Soc.* **43**, 2469.
—— —— (1922). *J. Amer. Chem. Soc.* **44**, 2926.
—— and Walker (1921). *J. Amer. Chem. Soc.* **43**, 2461.
Truszkowski (1928). *Biochem. J.* **22**, 767.
Willstätter and Schudel (1918). *Ber. deutsch. chem. Ges.* **51**, 780.
Wolff and Fernbach (1906). *Woch. Brau.* **23**, 743.

XLII. THE FORMATION OF SULPHATE FROM CYSTEINE AND METHIONINE BY TISSUES *IN VITRO*.

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In spite of the very large amount of work that has been done on the variations in the rate of excretion of sulphate by animals and men after the administration of various sulphur compounds, it seems that no one has shown that either an organ or a tissue preparation can make sulphate from an unoxidised sulphur compound *in vitro*. Rothera [1905] could find no sulphate when he incubated cystine with liver mince or when he perfused the liver with cystine, and it seems probable that several other workers have been similarly unsuccessful. The formation of sulphate from sulphuric esters by minced mammalian organs has been studied by Neuberg and Simon [1925], but this process, although perhaps connected with the type of oxidation which forms the subject of this paper, is obviously relatively simple.

The organ slice technique of Warburg [1923] is admirably suited to this work, for it causes little or no damage to the cells, and a series of experiments can be carried out under exactly comparable conditions, using parts of the same liver. By this means the troubles caused by the extreme individual variations between animals are avoided.

In this paper are described some preliminary experiments on the formation of sulphate by slices of kidney and liver. Later it is hoped that a larger group of substances will be investigated, as well as the probable intermediate stages in the oxidation and the extremely fragile enzyme systems by which these oxidations are carried out.

Experimental.

Rat tissue was used in all the experiments recorded; the animal was stunned and the required organs removed immediately and sliced as soon as possible. 30–80 mg. (dry weight) of slices were put in a vessel, of the type described by Krebs [1933], containing 8 ml. of bicarbonate Ringer solution. The latter was made up according to Krebs's directions [1932], except that magnesium chloride was used in place of magnesium sulphate. The vessels were filled with a gas mixture consisting of 95 % O_2 and 5 % CO_2 and shaken at 37°, generally for two hours. At the end of this time the flasks were taken out of the bath, rinsed on the outside with distilled water, dried round the joint with filter-paper and opened. The organ slices were removed with forceps, as much Ringer as possible was drained from them, and they were dried for 2–3 hours at 100° before being weighed. 2 ml. of 20 % trichloroacetic acid were added to the fluid in each flask, the mixture was poured into a 10 ml. pointed centrifuge-tube and the

protein precipitate centrifuged down. As a general rule the supernatant fluid was perfectly clear, but it sometimes contained small particles of tissue which floated because of entangled bubbles of CO_2. If the tube were shaken gently these could be centrifuged down.

Estimation of sulphate.

The method used for the estimation of sulphate is essentially that of Cuthbertson and Tompsett [1931]. In this the sulphate is precipitated in 70 % acetone with benzidine, the precipitate is washed thoroughly, and then the benzidine is estimated by diazotisation and coupling with thymol. Cuthbertson and Tompsett use a centrifuge throughout but very much more consistent results are obtained by using a sintered glass funnel (Schott 13f G4) of the type used by Pregl for the estimation of sulphur. The filter, mounted on a rubber bung, is placed inside a glass tube about 2 inches long and an inch wide, into the lower end of which the bung fits. This bung also carries a draining tube and an inlet tube for compressed air. The top of the wide glass tube can be closed firmly by another bung; fluid that is in the filter may then be forced through by opening the compressed air tube and closing the draining tube.

2 ml. of the centrifugate are added to 5 ml. of a 0·5 % solution of benzidine in acetone. The mixture is left in a stoppered tube for at least two hours on ice, it is then filtered by suction through the sintered glass funnel, and the tube is rinsed out on to the filter with acetone. The benzidine sulphate is washed by filling up the funnel (which holds 3 ml.) three times with acetone while it is connected with the pump. Care must be taken to wash the lip of the funnel during this process. The funnel is filled a fourth time, and the acetone is forced through by air pressure, the stem of the funnel being washed thoroughly with water during this last filtration. Two 3 ml. lots of boiling $N/3$ hydrochloric acid are used to transfer the benzidine sulphate to a test-tube; the fluid is forced through the filter by air pressure in each case. After sucking a little more $N/3$ HCl through the filter it is once more ready for use. The whole cycle of operations takes 6–10 minutes and the filter requires cleaning, by sucking nitric acid through it in the reverse direction, after about 10 estimations. At longer intervals it, like all the apparatus used in this work, is heated with cleaning mixture.

The benzidine is diazotised and coupled with thymol according to the directions of Cuthbertson and Tompsett except for the omission of the 15 % NaOH recommended by them[1]. The standard used was 0·3 ml. of a 0·053 % solution of benzidine hydrochloride in N HCl; this corresponds with 20γ of sulphur in the form of sulphate. The colorimeter cup containing the unknown solution is set at 20 on the scale, and the standard cup is adjusted to match; the standard scale now reads directly in thousandths of a mg. of sulphur in the form of sulphate in the 2 ml. sample of Ringer originally taken. On multiplying by five the figure that is recorded in the tables in this paper is obtained. All the estimations were done in duplicate, and the figure given is the mean of duplicates that did not differ by more than 1γ.

The cysteine used in this work was prepared by adding pyridine to an alcoholic solution of cysteine hydrochloride and washing the crystalline precipitate very thoroughly with alcohol. The glutathione, methionine and ergothioneine were prepared by the methods already published [Pirie, 1930; 1933, 1]. *S*-Ethylcysteine was prepared by the method of Clarke and Inouye [1931].

[1] I am very grateful to Miss E. Watchorn, of this laboratory, for pointing out the advantages of this modification.

Oxidation of cysteine and glutathione.

The results of a number of experiments on the oxidation of cysteine by liver slices are given in Table I. In each case the sliced liver, of which the dry weight is

Table I.

No. of rat	Wt. of cysteine in 8 ml. Ringer (mg.)	Dry wt. of liver (mg.)	Wt. of S found in the form of SO_4 (γ)
14	1·3	40	20
14	5·0	52	60
14	5·0	12	20
14	10·4	39	60
15	7	41	87 (72)
17	7	39	80
20	8	96	100
20	8 cystine	92	50
26	10	72	100
30	8	62	70
31	8	98	104 (100)
31	8 cystine	110	50
32	8	87	160
32	8	87	163

given, was suspended in 8 ml. of bicarbonate-Ringer containing the amount of cysteine specified. The flask was filled with the gas mixture and shaken for two hours at 37°. The figures in the fourth column are simply the amount of sulphur that was found in the flask in the form of sulphate at the end of the experiment; a series of blank experiments with cysteine alone, or with liver but no cysteine, or with liver slices that have been inactivated by heating to 60° or by prolonged freezing gave values ranging from 10 to 15γ. It is clear therefore that the sulphate concentration can be increased 5 or 10-fold by the oxidation of cysteine in this way. The reliability of the method used for the estimation of sulphate is also tested by the experiments done in nitrogen and by those in which different organs were used; these are described later.

The quantity of sulphate formed is too small to be estimated accurately by precipitation as barium sulphate followed by weighing. In a few cases, however, this has been attempted. A 4 ml. sample of the trichloroacetic acid centrifugate was used, and the weight of sulphur corresponding with the observed weight of barium sulphate was multiplied by 10/4; the figure so obtained is recorded in brackets in column 4 of certain of the tables. The two sets of figures are in sufficiently good agreement to make it certain that it is sulphate that is being estimated by the colorimetric method. It is necessary to emphasise this point, because the benzidine methods for the estimation of sulphate have often been criticised on account of their lack of specificity. Furthermore, some substances, cystine for example, are precipitated by the high concentrations of acetone used in the estimation. I find, however, that so long as sufficient trichloroacetic acid has been added in the first place, these precipitates can easily be washed free from adsorbed benzidine; they do not therefore interfere with the estimation. Small amounts of phosphate do not matter, but if it is present in large amounts sulphate estimations become impossible.

Table II contains the results of experiments in which cysteine is oxidised by kidney. Liver and kidney have much the same oxidising capacity, but the blood, testis, spleen, heart and lung of the rat seem to be quite inactive under these

Table II.

No. of rat	Wt. of cysteine mg.	Dry wt. of kidney mg.	Wt. of S found in the form of SO_4 (γ)
16	8	26	55
20	8	42	90
27	7	48	100
28	7	63	100
30	8	36	70
31	8	34	50
31	8	38	60 (50)

conditions. The study of the kinetics of sulphate formation from cysteine is complicated by the fact that under the conditions of these experiments the cysteine is being rapidly oxidised to cystine; this separates as minute crystals if the concentration exceeds about 1 mg. per ml. Although cystine at lower concentrations than this stays in solution it cannot be directly oxidised to sulphate. If cystine is shaken with tissue slices, some sulphate is indeed formed, but this is probably due to a preliminary reduction to cysteine by the systems which Hopkins and Elliott [1931] and Mann [1932] have described. Two experiments of this type are recorded in Table I. The cystine, dissolved in the minimum amount of dilute hydrochloric acid, was added to Ringer solution containing a corresponding amount of NaOH. No separation of free cystine could be observed.

If a number of flasks all containing the same quantities of cysteine and liver are set up, and the amount of sulphate present is estimated after various intervals, it is found that no more sulphate is formed after the nitroprusside reaction has disappeared. With 7–8 mg. of cysteine and 60–80 mg. dry weight of liver this generally takes from 60–90 minutes, but the time is variable, although the relationship is constant. The following experiment is more definite. 12 mg. of cysteine and 140 mg. dry weight of liver were shaken for an hour at 37° in 16 ml. of Ringer. The flask was then opened, 8 ml. of the fluid were withdrawn for sulphate estimation, and half the slices were taken out and placed immediately into another flask containing 6 mg. of cysteine in 8 ml. of Ringer, *i.e.* the same concentration of cysteine that the first flask, which now contains only cystine, had contained initially. Both flasks were then closed, refilled with gas mixture and shaken for an hour at 37°. The sulphate contents of each flask and of the fluid withdrawn from the first flask after one hour were estimated. Within the limits of the method the sulphate content of a 2 ml. sample was the same in each case. Clearly therefore no sulphate was formed during the second hour in the flask from which all the cysteine had disappeared, but a part of the same group of slices was very well able to make sulphate when supplied with fresh cysteine. This conclusion, that disulphides are first reduced to mercaptans before undergoing oxidation to sulphate, has already been reached by Lewis *et al.* [1924] and others from feeding experiments on the intact animal. Furthermore, cysteine but not cystine can undergo the further stage of oxidation by the disulphide form of thiocarbamide which I have already described [Pirie, 1933, 2].

Hele and Pirie [1931] and Schelling [1932] have found that the intact animal can oxidise glutathione to sulphate even if it is given subcutaneously. The figures in Table III show that glutathione is also oxidised by tissue slices. It is clear that kidney has a much greater capacity to oxidise glutathione than liver. This fact makes it probable that the first step is the hydrolysis of the tripeptide into its constituent amino-acids, for it is well known that kidney undergoes

Table III.

No. of rat	Wt. of glutathione mg.	Dry wt. of tissue mg.	Wt. of S oxidised to SO_4 (γ)
19	24	60 liver	30
19	15	60 liver	20
19	54	48 liver	65
19	54	43 kidney	105
19	9	43 kidney	68
21	30	64 liver	45
21	30	45 kidney	75
21	15	52 kidney	75
21	30	22 kidney	57
25	30	69 liver	40
25	30	29 kidney	50
25	60	30 kidney	65
25	20	44 kidney	65
25	10	44 kidney	55
29	21	56 liver	45

autolysis more rapidly than most tissues, and it is the only tissue in which Bierich and Kalle [1928] were able to find free cysteine. Confirmatory evidence can be got by applying Sullivan's test to the fluid in which liver or kidney has been shaken with glutathione. The former gives no trace of cysteine colour, *i.e.* there is less than 0·03 mg. in a 2 ml. sample, whereas the latter contains about 0·7 mg. The most probable explanation of this would seem to be that the peptide-splitting enzymes of kidney make cysteine more rapidly than the cysteine-oxidising enzymes can destroy it, whereas in the case of liver this is not so. Needless to say the fluid in which kidney has lain for two hours without the addition of glutathione gives no detectable Sullivan reaction.

Oxidation of methionine.

Both liver and kidney can oxidise methionine to very much the same extent. In both cases, however, the rate of oxidation is very much smaller than the corresponding rate in the case of a similar concentration of cysteine. These points are illustrated in Tables IV and V. It is clear that methionine has to be present

Table IV.

No. of rat	Wt. of methionine mg.	Dry wt. of liver mg.	Wt. of S found in the form of SO_4 (γ)
4	40	90	60
4	80	70	30
8	40	60	90
8	20	59	50
9	10	40	50
9	20	32	55
9	30	55	75
9	40	47	47
10	40	70	95
13	24	45	60
15	40	55	60
17	40	54	60
24	30	52	52
30	30	74	97
32	24	91	52
32	24	78	50

Table V.

No. of rat	Wt. of methionine mg.	Dry wt. of kidney mg.	Wt. of S found in the form of SO_4 (γ)
8	40	31	60
10	30	41	85
10	50	35	75
10	80	35	80
30	30	30	62

in twice or three times the concentration that is necessary in the case of cysteine to get an equal rate of formation of sulphate. Individual variation between rats makes comparisons difficult, but it would seem from the figures for rats 4 and 9 in Table IV that there is quite a definite optimum concentration for sulphate production from methionine. Evidence of this sort could not be obtained in the case of cysteine on account of the formation of cystine.

I have found it impossible to get consistent results in the presence of toluene or other disinfectants. It is exceedingly unlikely that the sulphate formation is due to bacterial action, and the relative constancy of the sulphate production in different experiments makes it still less probable. The figures in Table VI show that the amount of sulphate formed during the third hour of an experiment is the same as that formed during the first and second hours. This fact entirely excludes the possibility of bacterial action.

Table VI.

No. of rat	Wt. of methionine mg.	Dry wt. of liver mg.	Duration of experiment (hr.)	Wt. of S found in the form of SO_4 (γ)
12	30	87	0·5	20 (25)
12	30	57	1·0	36
12	30	52	2·0	60 (50)
12	30	49	3·0	85 (90)

Chase and Lewis [1933] have found in the urine of rats receiving *dl*-methionine a substance which gives a red colour with sodium nitroprusside in the presence of sodium cyanide. I have tested the fluid in which tissue slices have been shaken with methionine and find in all cases a faint but definite nitroprusside reaction. This reaction is given neither by fluid to which no methionine has been added nor by methionine solutions shaken without tissue. The possible significance of this will be discussed later.

Table VII.

No. of rat	Wt. of *S*-ethyl-cysteine (mg.)	Dry wt. of tissue (mg.)	Wt. of S found in the form of SO_4 (γ)
16	10	64 liver	15
16	40	76 liver	35
16	80	71 liver	57
16	40	32 kidney	37

In Table VII the results of an experiment with *S*-ethylcysteine are given. At a concentration at which cysteine would be very rapidly oxidised and methionine would be oxidised at an appreciable though not optimum rate this substance is hardly oxidised at all. It is only in relatively concentrated solutions that a definite formation of sulphate can be shown. The three substances cysteine, methionine and *S*-ethylcysteine do, however, form a series so far as *in vitro* oxidations are concerned which is very unlike their behaviour in the intact dog [Pirie, 1932]. There cysteine and methionine resembled one another while *S*-ethylcysteine was dissimilar. It is likely that this difference is due to

the fact that the tissue slice experiments are not complicated by the excretory mechanism, whereby an intact animal gets rid of foreign compounds into the urine.

Miscellaneous experiments.

Neither liver nor kidney can oxidise ergothioneine to sulphate under these conditions. This is somewhat unexpected since the sulphur atom in a thiol-glyoxaline is very much more readily oxidised to sulphate by chemical means than that in cysteine or methionine. Ergothioneine has been tested as a catalyst in the oxidation of the other sulphur compounds; here also no effect could be found. Thiocarbamide also was not oxidised by liver slices when present in low concentration, nor did it catalyse the oxidation of cysteine. It seems therefore that this oxidation cannot be directly compared with the oxidation by H_2O_2 [Pirie, 1933, 2]. The extract obtained by freezing and grinding liver and then centrifuging out the particles was also tested both as a substrate and as an activator; it was inactive. In this connection it should be recorded that the tissues of a rat from which food had been withheld for 24 hours had the usual oxidising power; rat 30 in Tables I, II, IV and V illustrates this.

Table VIII.

No. of rat	Wt. of substrate (mg.)	Dry wt. of liver (mg.)	Gas mixture	Wt. of S found in the form of SO_4 (γ)
17	None	53	95 % N_2 and 5 % CO_2	6 (20)
17	40 methionine	41		10
17	8 cysteine	49		15 (20)
17	30 methionine	46	95 % O_2 and 5 % CO_2	45
17	40 methionine	54		60
17	8 cysteine	39		80
17	30 methionine	53	Air	25
17	30 methionine	40	97·5 % O_2 and 2·5 % CO_2	45

Table VIII relates to the effect of different atmospheres on the oxidation by liver. The results call for little comment; as might be expected the oxidation does not proceed anaerobically. The oxidation is slow in air and there is no appreciable difference between the results obtained when a gas mixture containing 5 % CO_2 (p_H of Ringer 7·3) or one containing only 2·5 % CO_2 (p_H of Ringer 7·6) was used.

Little attempt has yet been made to investigate either the stability of this enzyme system or the effect of inhibitors upon it. Cooling to about $-5°$ generally inactivates the slices, and heating for 20 minutes at 60° does so with certainty, but short periods of freezing give erratic results. Finely ground tissue is inactive. The action of inhibitory substances is complicated by their effect on the autoxidation of the cysteine; a description of the effects observed will be published shortly.

Discussion.

Lawrie [1932] found that a rat, of the strain used in this laboratory, weighing about 240 g. excreted 10 mg. of sulphur in the form of sulphate per day when eating a synthetic diet. By feeding cystine the sulphate excretion could be raised to 20 mg. S per day, but at this level there was a definite rise in the neutral sulphur also. Now the liver and kidneys of a 240 g. rat weigh together about 12 g.; their dry weight is therefore 2·4 g., and this quantity of tissue will seldom be called on to oxidise more than 20 mg. of sulphur to sulphate in 24 hours. 50 mg. dry weight of tissue should on this basis oxidise 35 γ of sulphur to sulphate in 2 hours. The figures in Tables I and II show that tissue slices can make sulphate from cysteine at a rate considerably in excess of this if the cysteine

concentration is raised to unphysiologically high levels. Since, however, the sulphate-forming mechanism, when working *in vitro*, has to compete with the autoxidation processes this agreement is fairly satisfactory. The figures in Tables IV and V are more important, for methionine is the principal sulphur-containing constituent of most diets. When we consider the very high concentrations of methionine that are being used and also the fact that the action is proceeding in 95 % oxygen rather than in air, it is clear that there is little quantitative relation between the rates at which rat organs can oxidise methionine *in vitro* and *in vivo*.

It would be premature to put forward a theory of the mechanism of this oxidation with any degree of confidence. As a working hypothesis, however, the following scheme is suggested:

$$\begin{matrix} \text{R.SS.R} \\ \text{or} \\ \text{2R.SCH}_3 \end{matrix} \rightarrow 2\text{R.SH} \rightarrow 2\text{R.SOH} \rightarrow \text{R.SH} + \text{R.SO}_2\text{H} \rightarrow \text{H}_2\text{SO}_3 \rightarrow \text{H}_2\text{SO}_4$$

Of these processes the first and second are probably enzymic, the third proceeds spontaneously at body p_{H} [Pirie, 1933, 2], the fourth is enzymic and the fifth need not necessarily be so. The relatively slow rate at which sulphate is formed from methionine, taken in conjunction with the occurrence of disulphides in the fluid in which methionine is being oxidised, is at least consistent with the theory that this substance has to be demethylated before it can be oxidised.

Summary.

The oxidation of cysteine and methionine to sulphate by slices of rat liver and kidney has been studied.

Cystine can only be oxidised after undergoing reduction and glutathione only after hydrolysis.

Ethylcysteine is oxidised slowly and ergothioneine not at all.

A modification in the technique of micro-sulphate estimation is described.

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REFERENCES.

Bierich and Kalle (1928). *Z. physiol. Chem.* **175**, 292.
Chase and Lewis (1933). *J. Biol. Chem.* **101**, 735.
Clarke and Inouye (1931). *J. Biol. Chem.* **94**, 548.
Cuthbertson and Tompsett (1931). *Biochem. J.* **25**, 1237.
Hele and Pirie (1931). *Biochem. J.* **25**, 1095.
Hopkins and Elliott (1931). *Proc. Roy. Soc. Lond.* B **109**, 58.
Krebs (1932). *Z. physiol. Chem.* **210**, 33.
—— (1933). *Z. physiol. Chem.* **217**, 191.
Lawrie (1932). *Biochem. J.* **26**, 435.
Lewis, Updegraff and McGinty (1924). *J. Biol. Chem.* **59**, 59.
Mann (1932). *Biochem. J.* **26**, 785.
Neuberg and Simon (1925). *Biochem. Z.* **156**, 365.
Pirie (1930). *Biochem. J.* **24**, 51.
—— (1932). *Biochem. J.* **26**, 2041.
—— (1933, 1). *Biochem. J.* **27**, 202.
—— (1933, 2). *Biochem. J.* **27**, 1181.
Rothera (1905). *J. Physiol.* **32**, 175.
Schelling (1932). *Amer. J. Physiol.* **102**, 714.
Warburg (1923). *Biochem. Z.* **142**, 317.

XLIII. THE CONSTITUTION OF COCCERIN.

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Introduction[1].

Liebermann [1885] showed that boiling benzene extracted an alcohol-insoluble wax which he called coccerin from the cochineal insect (*Coccus cacti*). By saponification with strong alcoholic potash and separation of the acids as calcium soaps he obtained an alcohol, cocceryl alcohol, which melted at 101–104°, and an acid, coccerinic acid, which melted at 92–93°. Two years later Liebermann and Bergami [1887] investigated the structure of these two compounds. Analysis of cocceryl alcohol suggested that it was a C_{30}-alcohol with two oxygen atoms. Acetylation with acetic anhydride in a sealed tube at 170° gave a product, M.P. 48–50°, apparently a diacetate; similar evidence was obtained on benzoylation, and it was concluded that cocceryl alcohol was a dihydric alcohol $C_{30}H_{60}(OH)_2$. On oxidation with chromic acid it gave an acid M.P. 59–60° which was identified as pentadecanoic acid. Analysis of the coccerinic acid, and of its salts, suggested that it was a hydroxy-acid $C_{31}H_{62}O_3$, although no evidence for the hydroxyl group was obtained by acetylation or benzoylation. This acid on oxidation was also stated to give pentadecanoic acid, which was identified as its methyl ester, M.P. 66–68°. As coccerin gave by saponification two parts of acid to one of alcohol it was considered to be $C_{30}H_{60}(C_{31}H_{62}O_3)_2$, in keeping with the dihydric nature of the alcohol.

The wax has been more recently examined by Becker [1931], who recrystallised it many times from benzene and chloroform, thereby raising the M.P. to 104–105°. By saponification with $N/2$ alcoholic potash and separation of the calcium soaps he obtained approximately equal amounts of alcohol, M.P. 102°, and of acid, M.P. 94–95°. His analysis suggested that the alcohol was $C_{32}H_{66}O_2$, and on acetylation in the usual way with acetic anhydride and fused sodium acetate it gave a monoacetate, M.P. 78–79°. Treatment in a sealed tube with acetic anhydride at 170° gave a mixture from which only the monoacetate could be recovered. His analysis of coccerinic acid agreed better with the formula

[1] The melting-points below 110° (except those of oximes) recorded in this paper were obtained by the method described by Piper *et al.* [1931] and are corrected.

The nomenclature used in this paper is based on the following system, which has recently been made official (permissive) by the International Union of Chemistry [*cf. J. Chem. Soc.* (1931), 1610].

$R.CH_3$	$R.CH_2OH$	R.CHO	$R.CO_2H$	R.COCl	$R.CONH_2$	$R.CH_2NH_2$
-ane	-anol	-anal	-anoic acid	-anoyl chloride	-anoamide	-anamine

The number will be placed immediately before the suffix to which it refers, and the C of the CO_2H group will be numbered 1. The system is more logical than the one previously used in publications from this laboratory and will be adopted in future. A. C. C.

$C_{32}H_{64}O_3$, and accepting Liebermann and Bergami's isolation of pentadecanoic acid, he concluded that cocceryl alcohol and coccerinic acid had corresponding structures. Coccerin therefore had the same biological origin as the plant waxes, and its components were formed by a Cannizzaro reaction from two molecules of an aldehyde built up by repeated aldol condensation.

In connection with the research which is being carried out in this laboratory into the metabolism of waxes this cocceryl alcohol was of interest. Since pentadecanoic acid is now known to melt at 54° and its methyl ester at about 19° we were not prepared to accept all Liebermann's conclusions; in particular Becker's failure to obtain a diacetate suggested to us that the second oxygen atom might be ketonic. Clemmensen reduction might therefore provide us with *n*-dotriacontanol, an alcohol which would be extremely useful in connection with the X-ray analytical work which we are undertaking in conjunction with Dr Piper.

Our first preparation of the wax was made from 15 kg. of cochineal by Liebermann's method. By saponifying in strong benzene-alcoholic potash and separating the acids as their calcium soaps there were obtained approximately equal amounts of alcohol and acid, confirming the observations of Becker. The alcohol after many recrystallisations from benzene and alcohol melted at 99–100°. A typical example of one out of many analyses confirmed the data of both Liebermann and Becker, *viz.* C, 79·5 % and H, 13·2 %. On acetylation with acetic anhydride in the presence of fused sodium acetate we obtained a monoacetate, again confirming Becker's results. X-ray analysis gave a good photograph, 23 orders measuring 90·4 Å. At the time we could not be certain of the chain length, but we were able to deduce two facts: (1) if the chain length were about 32 carbon atoms, then the doubled spacing showed that the alcohol group was attached to the last or penultimate carbon atom; (2) the intensity distribution showed that another polar group was attached at some point in the carbon chain.

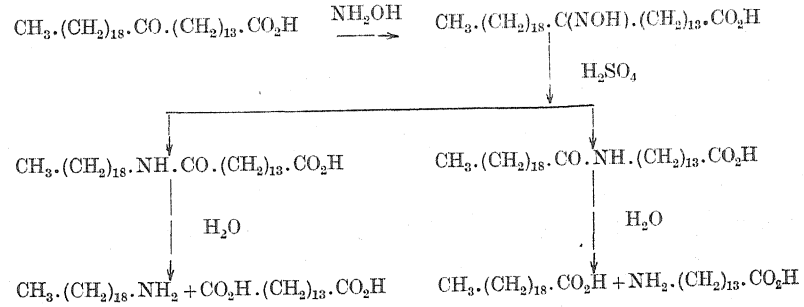
Treatment with hydroxylamine readily gave an oxime, M.P. 77·5–79°, confirming our surmise that the second oxygen atom was ketonic. Clemmensen reduction gave a monohydric alcohol, M.P. 89·7°, and this on reduction *via* the iodide a paraffin. The melting-point and transition temperatures of the latter showed that it was slightly impure *n*-tetratriacontane, and this was confirmed by X-ray analysis, which gave both the A and C spacings, and we know that the presence of the C spacing is specific [Piper *et al.*, 1931]. X-ray analysis of the monohydric alcohol gave a fair picture, 11 orders measuring 90·2 Å. At this stage in our research the melting-points and crystal spacings for the *n*-fatty acids and primary alcohols with more than 26 carbon atoms were not known, but by extrapolating the data obtained by Malkin [1930] from the primary alcohols with 12 to 25 carbon atoms this spacing gave a reasonably good agreement for the primary alcohol with 34 carbon atoms. Cocceryl alcohol was therefore a keto-*n*-tetratriacontanol, which requires C, 80·2 % and H, 13·5 %. These values were appreciably higher than we and the above-mentioned authors had obtained, and it was clear that some oxygen-rich impurity was present, because a small amount of unsaponified wax would not lower the carbon content. Repeated attempts to purify the alcohol and its acetate with various solvents were not very successful.

Another preparation of wax from 15 kg. of cochineal was obtained, and this was submitted to repeated extraction with large volumes of benzene-alcohol (1:5). After the tenth extraction the residue melted sharply at 106° and its weight was only about one-third that of the original wax. This was saponified first

with benzene-alcoholic potash and then with sodium ethoxide, the acids in both cases being converted into the calcium soaps. The cocceryl alcohol thus obtained melted sharply at 100–100·2°, and on analysis gave, within experimental error, the correct figures, *viz.* C, 80·0 %, H, 13·5 %. Although this procedure undoubtedly removed the impurity it was wasteful, and as we were desirous of collecting a large quantity of both cocceryl alcohol and the acids for further analysis we repeated it with another batch of wax (from 30 kg. of cochineal) at the same time paying attention to the products extracted by the benzene-alcohol, which were finally collected into two fractions. Full details are given in the experimental section. The results show clearly that extraction with hot benzene-alcohol does not *per se* effect very much purification. As will be discussed later, the so-called coccerinic acid of Liebermann is a mixture of *n*-fatty acids and a ketonic acid, so that coccerin is a mixture of true waxes whose solubility in hot benzene-alcohol will naturally depend on the chain-length of the particular acid component. The impurity present is undoubtedly stable to saponification with alcoholic potash, but is converted into water-soluble products by saponification with sodium ethoxide, and it is the omission of this essential step which has led to the misleading conclusions drawn from earlier work.

The purified cocceryl alcohol on Clemmensen reduction gave a monohydric alcohol, M.P. 92·2°, which was shown to be *n*-tetratriacontanol by (1) reduction *via* the iodide to *n*-tetratriacontane, M.P. 72·6–72·8°, and (2) oxidation to *n*-tetratriacontanoic acid, M.P. 98·3–98·5°; the purity of all three products was confirmed by X-ray analysis.

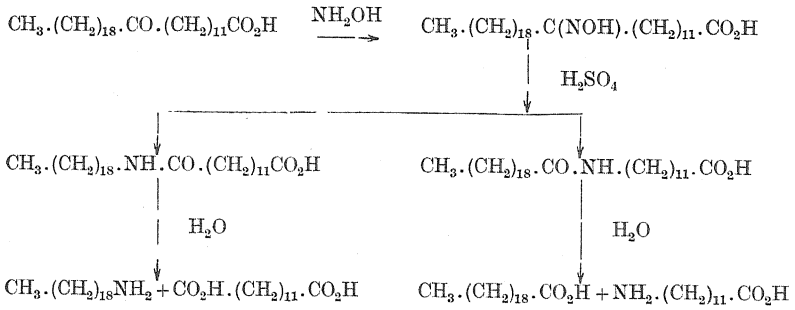
Oxidation of the keto-alcohol with chromium trioxide in glacial acetic acid gave the corresponding ketonic acid in good yield. This was treated with hydroxylamine and the resulting oxime converted into the corresponding amides by heating with concentrated sulphuric acid. These amides were hydrolysed by heating with hydrochloric acid and the products thus obtained were identified as *n*-nonadecanamine, *n*-eicosanoic acid, *n*-tridecane-(1:13)-dicarboxylic acid and 14-aminomyristic acid. From this it follows that the ketonic acid is 15-keto-*n*-tetratriacontanoic acid and that the above products are formed as follows:



Cocceryl alcohol is therefore 15-keto-*n*-tetratriacontanol.

The acids obtained from the three fractions of the wax, the so-called coccerinic acid of Liebermann, showed variations in melting-point suggesting that they were mixtures similar to those met with as components of plant waxes. Analysis for carbon and hydrogen however again showed the presence of excess oxygen above that required for appropriate mixtures of *n*-fatty acids. Each fraction was unaffected by acetylation, showing that the acids were not hydroxy-acids,

as had been suggested by Liebermann. But treatment with hydroxylamine gave a product freely soluble in cold acetone which could be readily separated from an insoluble residue. This soluble product on purification melted in each case at 56–57°, and analysis showed that it was a ketoxime. Hydrolysis regenerated the ketonic acid, which melted at 104·5–105°. This was shown to be a keto-*n*-dotriacontanoic acid by Clemmensen reduction to *n*-dotriacontanoic acid. The position of the ketonic group was determined as before by heating the ketoxime with sulphuric acid to effect a Beckmann transformation. The resulting amides were hydrolysed with hydrochloric acid, and the products thus obtained were identified as *n*-nonadecanamine, *n*-eicosanoic acid, brassylic acid and 12-aminolauric acid. From this it follows that the ketonic acid is 13-keto-*n*-dotriacontanoic acid, and that the above products are formed as follows:



The constitution has been confirmed by synthesis.

The fraction of the acids insoluble in cold acetone was again treated with hydroxylamine to remove any residual ketonic acid and the product finally obtained recrystallised twice from acetone at 37°. This melted at 93·6° and was shown to be *n*-triacontanoic acid.

It will be clear from the above results that the "coccerinic acid" of Liebermann was a mixture, and as correctly the name should be applied to the acid corresponding to cocceryl alcohol, *i.e.* 15-keto-*n*-tetratriacontanoic acid, which has not yet been found in natural products, we recommend that it be abandoned.

This elucidation of the structure of coccerin is of great interest from the point of view of wax metabolism, for not only do the ketonic alcohol and ketonic acid provide a clear case of β-oxidation, but they both contain the group $CH_3.(CH_2)_{18}.CO$, which corresponds with the group $CH_3.(CH_2)_{18}.CHOH$ present in *n*-nonacosan-10-ol, a constituent of apple cuticle wax [Chibnall *et al.*, 1931]. Further discussion is reserved for a later paper.

The difficulty which we experienced in the early stages of this research in purifying coccerin and its constituent alcohol and acids has forcibly drawn our attention to two steps in the procedure which we now consider to be essential. In the first place the initial saponification of the wax with alcoholic potash should be followed by saponification with sodium ethoxide. This is a usual precaution if esters of sterols are known to be present, but it is necessary in the present case in order to remove more effectively impurities which are stable to boiling alcoholic potash. In the second place the alcohol and acid liberated by saponification cannot be effectively separated unless the acid is converted into the very insoluble calcium soap. Liebermann adopted this procedure, as also have certain more recent workers, but others have omitted it probably because it has not been generally realised that the potassium or sodium soaps of the

fatty acids with carbon chains longer than 26 become increasingly soluble in hot acetone or hot benzene with increase of chain length. For these two reasons we consider that most, if not all, of the previous work on the constitution of insect waxes is in need of reinvestigation, and it is to be regretted that in most cases the waxes themselves are so difficult to obtain even in small amounts.

This applies especially to the wax of *Pemphigus xylostei*, which has been recently investigated by Schulz and Becker [1931]. The insects were collected over a period of many years and gave 1·9 g. of wax. This was repeatedly crystallised from chloroform-alcohol, and the sharp melting-point, 108–108·5°, to which they call attention, suggests that extraneous material had been successfully removed. The wax was then saponified with benzene-alcoholic potash, the alkali removed by washing with water and the alcohol (pemphigus alcohol) separated from the acid (pemphigus acid) by extraction with hot benzene. The pemphigus alcohol was recrystallised many times from hot alcohol, but the indefinite melting-point (100–105°) shows clearly that the material was still contaminated with a small amount of potassium soap. Carbon and hydrogen analysis of the alcohol and of its acetate (M.P. 75–77°) suggested that it was a C_{34} dihydric alcohol, the higher homologue of cocceryl alcohol. Accepting Liebermann's isolation of pentadecanoic acid after oxidation of the latter they suggest that pemphigus alcohol is 18-hydroxytetratriacontanol. We do not agree with these conclusions. Their analysis of the alcohol (C, 80·0, 80·21; H, 13·46, 13·50°) agrees much better with that of a ketotetratriacontanol (C, 80·2; H, 13·5 %) than with that of a hydroxytetratriacontanol (C, 79·9; H, 13·8 %). The melting-point (75–77°) of their acetate suggests to us the presence of only one acetyl group (*cf.* the acetate of cocceryl alcohol, M.P. 80·9–81·3°); they infer that it is a diacetate from the result of a single analysis for carbon and hydrogen on a very small amount of material. To obtain collateral evidence we have ourselves reduced cocceryl alcohol to 15-hydroxy-*n*-tetratriacontanol, and as we expected, the diacetate has a much lower melting-point (50–51°). Pending reinvestigation of this wax therefore we prefer to consider that pemphigus alcohol is a ketotetratriacontanol, and that in all probability it is identical with cocceryl alcohol. The melting-point of pemphigus acid (101–102°) is too low for a hydroxy- (or keto-) acid with 34 carbon atoms as they suggest, and it is undoubtedly a mixture similar to "coccerinic acid" but containing a small proportion of *n*-fatty acid, so that the melting-point of the pemphigus wax (108–108·5°) should be higher than that of coccerin (106–106·5°).

Experimental.

Preparation of the wax. 30 kg. of cochineal were extracted with boiling benzene by British Drug Houses, Ltd. The resulting extract, from which the main bulk of the solvent had already been removed before it was delivered to the laboratory, was boiled for 15 minutes with an equal volume (5100 ml.) of benzene and the mixture allowed to stand until cold. The product which separated was filtered off and recrystallised from 3 litres of boiling benzene. The weight of crude wax, dried in an air-oven at 90°, was 149 g., and the M.P. indefinite 99–102°.

Separation of the wax into three fractions. The crude wax was first boiled with 2 litres of 95 % alcohol for 6 hours and the mixture filtered hot. The residue melted at 103–104° and the material (2·5 g.) recovered from the solvent at 98–98·5°. The residue was then extracted seven times successively for 1 hour with 2 litres of boiling benzene-alcohol (1 : 4), the mixtures being filtered hot through a conical filter under slight suction. The residue melted at 105–106°, while the material recovered from the last hot filtrate melted at 100–101°. The total amount of material recovered from these first eight extractions (Fraction 1), weighed 35 g. Seven further extractions with similar amounts of benzene-alcohol were then made. The material recovered from these extracts (Fraction 2) weighed 34·9 g. and melted at 103–103·5°. The final residue (Fraction 3) weighed 62·0 g. and melted at 106–106·5°. A further 9·7 g. of crude wax were recovered from the numerous filter-papers used in the above separation and were neglected.

Saponification of the wax. The treatment of Fraction 3 given below applies equally to Fractions 1 and 2. The wax (62 g.) was dissolved in 1800 ml. of hot benzene, 1200 ml. of 10 % alcoholic potash were added, and the mixture was boiled under reflux for 12 hours. At the end of that period 3 litres of 95 % alcohol containing 160 g. of calcium chloride were added and the boiling continued for another 2½ hours. The mixture was then filtered hot, using a battery of hot-water-jacketed conical filters. The residual calcium soaps were extracted three times successively with 3 litres of boiling alcohol and then with the same volume of boiling acetone. The filtrates were cooled, and the crude cocceryl alcohol which separated was collected. This material (41 g.) was treated five times successively with boiling acetone and filtered hot. The filtrates on cooling gave 24 g. of crude alcohol. The residue insoluble in the hot acetone (12 g.) contained calcium soaps, which in the previous stage must have been rendered soluble in hot alcohol by the excess of potash present, a little unsaponified wax and much unidentified material. The crude alcohol and residue were therefore collected, dissolved in 1200 ml. of hot benzene and boiled under reflux for 3½ hours with sodium ethoxide (20 g. of sodium in 480 ml. of alcohol). 30 g. of calcium chloride in 250 ml. of 95 % alcohol were then added, and the boiling was continued for 2 hours. The mixture was filtered hot, and the insoluble soaps were extracted three times successively with 1 litre of boiling 95 % alcohol and finally with 1 litre of boiling acetone. The combined filtrates on cooling gave 26·5 g. of crude cocceryl alcohol, M.P. 99–100°. This was digested with 3½ litres of boiling acetone and filtered hot. The filtrate on cooling gave 15 g. of alcohol, M.P. 99·6–99·8°. The residue was treated three times successively in the same way, giving a further 4·7 g. of alcohol, M.P. 100·2–100·5°. The ultimate residue of soap weighed 0·8 g., and the acetone mother-liquors on evaporation gave 4 g. of crude alcohol which was neglected.

The calcium soaps from the first saponification were boiled under reflux with 2 litres of glacial acetic acid until the solution was clear and the mixture poured into an excess of cold water. The precipitated acids were collected, the treatment with glacial acetic acid repeated and the product crystallised from acetone. The material thus obtained (25·7 g.) was digested for some time with 3 litres of boiling acetone and filtered hot. On cooling, the filtrate gave 14·1 g. of fatty acid, M.P. 98·2–99°. Three further extractions of the residue gave in all 6·5 g. of fatty acid, M.P. 104–105°. The ultimate residue was neglected, as also were the 3 g. of crude acid recovered from the acetone mother-liquors on evaporation. The gross yield of acids recovered was 23·6 g. and of alcohol 23·7 g., showing a loss of 14·7 g.

Fraction 2 on similar treatment gave 13·1 g. of crude acids and 11·0 g. of crude alcohol, showing a loss of 10·8 g. The crude acids on digestion with hot acetone gave 11·6 g. of material, M.P. 98·0–98·4°, and the crude alcohol 9 g. of material, M.P. 99–100°. Fraction 1 in the same way gave 12·5 g. of crude acid and 11·0 g. of crude alcohol, showing a loss of 11·5 g. The acetone treatment gave 11 g. of acid, M.P. 89–90°, and 9 g. of alcohol, M.P. 100–101°. In all three fractions the major part of the loss occurred during the saponification with sodium ethoxide.

Fractionation of the fatty acids. The two samples of acid from Fraction 1, and the samples from Fractions 2 and 3 were separately treated with hydroxylamine in the following way. The acid (14·1 g.) in 800 ml. of alcohol was boiled under reflux for 8 hours with 6 g. of hydroxylamine hydrochloride and 9 g. of potassium hydroxide. The mixture was then poured into 6 litres of dilute sulphuric acid, the separated material filtered off and washed thoroughly with

water. It was then taken up in 1300 ml. of warm acetone; 300 ml. of ether were stirred in, and the mixture was allowed to stand for some hours at room temperature. 4·35 g. of *n*-fatty acid crystallised out and were filtered off. The other three samples gave in the same way 0·93, 6·35 and 1·8 g. of crystalline *n*-fatty acid, which were further treated as described later.

The acetone-ether mother-liquors were reduced to one-eighth of their original volume and cooled in the ice-chest. The material which separated was filtered off and dissolved in 1 litre of hot acetone. On cooling to room temperature a first crop of crude oxime weighing 6·4 g. was obtained. The mother-liquor was concentrated to 200 ml. and cooled in the ice-chest. A second crop of crude oxime was thus obtained. The latter was repeatedly extracted at room temperature with changes of cold acetone (shaking and centrifuging) until only a small residue remained undissolved. On evaporation of the solvent the oxime was recovered, and the cold extraction was repeated. The oxime (12·75 g.) then crystallised out in rosettes of white needles.

Constitution of the ketonic acid.

Preparation of the ketonic acid from the ketoxime. The ketoxime melted at 56–57°. (Found: C, 75·5; H, 12·5; N, 2·4 %. $C_{32}H_{62}O_2.NOH$ requires C, 75·4; H, 12·5; N, 2·7 %.) The regeneration of the ketonic acid from the ketoxime was found to be unexpectedly difficult, owing presumably to a small amount of amide formation. The following procedure was tedious and wasteful, and we are hoping that further research will enable us to increase the yield materially. 9·8 g. of ketoxime were warmed on the water-bath with 100 ml. of formaldehyde (40 %), and then 100 ml. of concentrated hydrochloric acid were added with stirring [Lapworth, 1907]. Separation of solid material rapidly took place and appeared to be complete in about 2 minutes. After remaining on the bath for about 15 minutes the mixture was poured into water, filtered and the residue washed with water and then crystallised from acetone. The material, which was recovered in quantitative yield, melted at 99·5–100°. After a prolonged fractionation from acetone at 37° there were finally obtained 1·9 g. of the ketonic acid, M.P. 104·5–105°. (Found: C, 77·9; H, 12·7 %. $C_{32}H_{62}O_3$ requires: C, 77·7; H, 12·6 %.) The lowest-melting fraction obtained melted at 96·4–96·7° and contained about 0·2 % of N (Dumas).

Conversion of the oxime of the ketonic acid into the amides. The oxime (4·4 g.) was heated on the water-bath with concentrated sulphuric acid (25 ml.) for $2\frac{1}{2}$ hours. The brown solution was cooled and slowly poured on to crushed ice, and the precipitated amides were collected and crystallised first from 250 ml. of glacial acetic acid and then from an equal volume of acetone. The product obtained was a faintly pink crystalline powder melting at 102°.

Hydrolysis of the above amides. 2·5 g. of the amides were heated with 12 ml. of concentrated hydrochloric acid in a sealed tube at 180° for 4 hours. The resulting dark brown products were mixed with 100 ml. of water and distilled in a current of steam. No volatile acids were separated. The aqueous mixture was therefore filtered and the insoluble material taken up in hot alcohol and filtered to remove charred products. The filtrate was concentrated to 50 ml. and 2 volumes of ether were added. On standing overnight a mass of thin white crystalline flakes separated, which were filtered off (Fraction A; wt. 0·29 g.). The filtrate was concentrated to 10 ml.; 20 ml. of hot benzene were added and the mixture allowed to stand overnight. In the morning a further 30 ml. of benzene were added with stirring and the white solid which separated was filtered off (Fraction B; wt. 0·38 g.). The mother-liquor was taken to dryness and the

residue dissolved in 60 ml. of hot alcohol. The solution was made alkaline to phenolphthalein with alcoholic sodium hydroxide and allowed to stand overnight. A white powder separated and was filtered off (Fraction C; 0·45 g.). The mother-liquor on evaporation gave Fraction D.

Fraction A. This was crystallised from 40 ml. of hot benzene, and gave 0·26 g. of material which was then repeatedly extracted with ether. The insoluble residue was dissolved in 20 ml. of alcohol and 2 volumes of ether were added. The mass of thin colourless plates which separated overnight was filtered off and washed with ether. The M.P. was indefinite and analysis suggested that the substance was *n*-nonadecanamine hydrochloride. (Found: C, 71·3; H, 13·3; N, 4·2 %. $C_{19}H_{41}N.HCl$ requires C, 71·3; H, 13·2; N, 4·4 %.) For confirmation this amine, which has not hitherto been prepared, was synthesised from pure *n*-eicosanoic acid by the method of Jeffreys [1899]. The synthetic amine and the substance from Fraction A both gave benzoates crystallising from alcohol in fine needles melting sharply at 88° either alone or when mixed. Samples of *n*-octadecanamine hydrochloride and *n*-eicosanamine hydrochloride gave benzoates melting at 88·2° and 91·5° respectively, which were depressed when mixed with the benzoate from Fraction A.

Fraction B. This was extracted several times with boiling benzene and the insoluble residue crystallised from 20 ml. of 2*N* hydrochloric acid. A first small crop of crystals separated in a few hours and was filtered off. These melted at 145° and were found impossible to purify. The filtrate on long standing gave a second crop of material in the form of fine colourless lamellae. These melted at 163°, alone and when mixed with a sample of authentic 12-aminolauric acid hydrochloride prepared by the late Dr Le Sueur. (Found: (micro-Dumas) N, 5·6%. $C_{12}H_{25}O_2N.HCl$ requires: N, 5·6%.) The micro-Kjeldahl method gave a low result, possibly owing to ring formation. The free amino-acid was obtained by dissolving the hydrochloride (18 mg.) in the calculated volume of dilute sodium hydroxide solution. The white crystalline mass which separated on cooling was collected, washed with cold water and dissolved in boiling water (10 ml.) from which it crystallised on cooling in large thin plates. The M.P. was 183° (uncorr.), unchanged when mixed with a sample of authentic 12-aminolauric acid prepared by the late Dr Le Sueur (M.P. 183°). (Found: (micro-Dumas) N, 6·5 %. $C_{12}H_{25}O_2N$ requires: N, 6·5 %.)

Fraction C. This was dissolved in 30 ml. of glacial acetic acid to decompose the soaps and poured into water. The solid which separated was filtered off and crystallised from ice-cold acetone. Three crops were obtained melting at 66°, 72·5° and 70° respectively. The first of these was extracted several times with boiling light petroleum (B.P. < 40°) and the extracts were concentrated to 10 ml. The crystalline material which separated melted at 73°. This and the second and third crops mentioned above were collected (0·15 g.) and recrystallised twice from 10 ml. of 90 % alcohol. The final product melted at 74–74·2°, and analysis showed that it was *n*-eicosanoic acid. (Found: C, 77·2; H, 12·9 %; mol. wt. by titration 313. $C_{20}H_{40}O_2$ requires: C, 76·9; H, 12·9 %; mol. wt. 312.) A mixed M.P. of 74·4–74·8° was obtained with a sample of synthetic *n*-eicosanoic acid, M.P. 75·0–75·2°, which had been purified by fractional distillation of the ethyl ester. In a mixed melt with synthetic *n*-heneicosanoic acid, M.P. 74·2–74·6° the M.P. was depressed to 70·5°.

Fraction D. This was treated with ether and allowed to stand overnight. The solid which separated was filtered off and the ethereal filtrate extracted with water. The solid was dissolved in this aqueous extract, which was then acidified and again extracted with ether. The ethereal solution yielded 0·23 g.

of solid, which was extracted repeatedly with small quantities of light petroleum (B.P. < 40°). The residue was recrystallised twice from benzene, then from acetone (charcoal) and again from benzene. The product (0·09 g., M.P. 108°) was dissolved in 150 ml. of boiling water, which was filtered hot. Fine white needles separated on cooling (M.P. 111·5°). These were collected, dissolved in 30 ml. of boiling absolute alcohol and titrated hot to phenolphthalein with alcoholic sodium hydroxide. After standing some days small plates of the sodium soap crystallised out. These were filtered off and dissolved in 50 ml. of boiling dilute hydrochloric acid. The crystals which separated on cooling were shown to be brassylic acid. (Found: C, 64·3; H, 10·0 %; mol. wt. by titration (dibasic acid) 250. $C_{13}H_{24}O_4$ requires: C, 63·9; H, 9·9 %; mol. wt. 244.) The M.P. was 113°, unchanged when mixed with authentic brassylic acid (M.P. 113·5°).

*Synthesis of 13-keto-*n*-dotriacontanoic acid.* The method of Robinson [1930 and unpublished data] was used. Ethyl 11-bromoundecanoate was condensed with ethyl sodioacetoacetate to give ethyl 2-acetylbrassylate. Ethyl sodio-2-acetylbrassylate (from 4 g. of the ester) in anhydrous ether was added slowly to *n*-eicosanoyl chloride (from 2·8 g. of the acid) in anhydrous ether and the mixture allowed to stand overnight. The next morning it was boiled to complete the reaction, the ether removed and the condensation product extracted with 50 ml. of alcohol. 3 litres of 3 % alcoholic (95 %) sodium hydroxide were then added, and the mixture shaken for 10 hours on two successive days. Half the alcohol was then removed by evaporation and the remainder boiled under reflux for 1 hour. After cooling in the ice-chest the precipitated soaps were filtered off, decomposed by boiling with aqueous hydrochloric acid and the free acids crystallised first from acetone and then from glacial acetic acid. The yield of crude acid was 1·1 g., M.P. 99·5–100·5°. To remove residual *n*-eicosanoic acid the material was recrystallised three times successively from acetone at 37°. The melting-point was 104·5–105°, unchanged when mixed with the cochineal acid. (Found: (Schoeller) C, 77·7; H, 12·6 %. $C_{32}H_{62}O_3$ requires: C, 77·7; H, 12·6 %.) The oxime was prepared in the usual way and crystallised repeatedly from acetone. The melting-point was 58–59°, and 57–58° when mixed with the oxime of the cochineal acid, which melted at 56–57°.

Constitution of the n-*fatty acids.*

The four samples of *n*-fatty acids obtained during the fractional crystallisation of the ketoxime were collected and 12 g. again treated with hydroxylamine as before. 1·5 g. of crude oxime and 9 g. of *n*-fatty acid, M.P. 92·8–93°, were obtained. The *n*-fatty acid was recrystallised twice from acetone at 37°, when the melting-point was raised to 93·4–93·6°, unchanged on further treatment. It was *n*-triacontanoic acid. (Found: C, 79·5; H, 13·3 %; mol. wt. by titration 454, 453. $C_{30}H_{60}O_2$ requires: C, 79·6; H, 13·4 %; mol. wt. 452·5.) The mixed melt with pure *n*-triacontanoic acid (M.P. 94°) was 93·6–93·8°. The X-ray photograph (Series B, 271) gave 32 orders in the B form measuring 71·1 Å., as against 71·4 Å. standard. There was a trace of the C spacing in the 3rd order showing that the acid probably contained less than 1 % either of higher *n*-fatty acids or of the keto-acid which had escaped oximation. This is the first recorded isolation from natural sources of *n*-triacontanoic acid as distinct from "melissic acid," which melts at 90–91° and has been considered by various workers to be a normal or *iso*-fatty acid with either 30 or 31 carbon atoms. We shall deal with the constitution of this acid in a later paper.

Constitution of cocceryl alcohol.

The samples of cocceryl alcohol obtained from the three fractions mentioned above were purified by treatment with acetone in the following way. The material (37·7 g.) was stirred with 1200 ml. of acetone at 40° for a short time and filtered hot. The residue was recrystallised from boiling acetone and the product thus obtained again treated with 1200 ml. of acetone at 40°. After the fourth repetition of this operation the M.P. of the final residue (26 g.) was constant at 100·5–100·7°. Treatment of the acetone mother-liquor gave several fractions melting between 99° and 100° which have not yet been further purified.

Properties of cocceryl alcohol. The pure alcohol crystallises from most organic solvents, and particularly from acetone at 37°, in small colourless rhombic leaflets which melt sharply at 100·5–100·7° and set with a nucleus in long needles at 99·7°. (Found: C, 80·0, 80·0; H, 13·5, 13·4 %. $C_{34}H_{68}O_2$ requires: C, 80·2; H, 13·5 %.) It is insoluble in all organic solvents in the cold, is slightly soluble in boiling ether and low-boiling petroleum and is readily soluble in boiling benzene or boiling acetone.

Treatment with acetic anhydride and fused sodium acetate gave the *monoacetate*, M.P. 80·9–81·3°, which is soluble in ether and to a small extent in cold acetone. (Found: C, 78·3; H, 12·7 %. $C_{36}H_{70}O_3$ requires: C, 78·5; H, 12·8 %.) To find out if the presumed diacetate of Liebermann and Bergami [1887] was obtained through enolisation of the ketonic group we prepared the acetate according to their directions. 1 g. of the alcohol was heated with 0·5 g. of acetic anhydride in a sealed tube at 170° for 2 hours. The resulting product crystallised from ether-acetone in fine rhombic plates melting at 80·9–81·2° and was the monoacetate.

The *oxime*, prepared in the usual way and crystallised from alcohol, melted at 74·5–75°. (Found: C, 77·4; H, 13·5; N, 2·5 %. $C_{34}H_{68}O.NOH$ requires: C, 77·9; H, 13·3; N, 2·7 %.) It is readily soluble in cold ether and sparingly so in cold acetone.

Reduction of cocceryl alcohol to n-*tetratriacontanol.* 17·7 g. of cocceryl alcohol were reduced by Clemmensen's method [Le Sueur, 1915] using 160 g. of amalgamated zinc (2 portions) and 160 ml. of concentrated hydrochloric acid (changed six times). The reaction was allowed to proceed for 60 hours, when the acid was diluted with water and the reduced alcohol, together with the residual zinc, filtered off. The mixed product was treated with a large volume of boiling acetone, which was filtered hot. On cooling the filtrate gave 14·8 g. of reduced alcohol, M.P. 88·5–89·0°. A second treatment with hot acetone gave a further 3·2 g., M.P. 90·9–91·3°. The lower-melting fraction was recrystallised twice from acetone at 37° which raised the melting-point to 91·2–91·5°. The products were then combined and 10 g. acetylated with 12·8 g. of fused sodium acetate and 300 ml. of acetic anhydride. The acetate was washed with water, crystallised from acetone and then distilled. The bath temperature was 305°, and 6 fractions were collected at 220–235°/0·004 mm. On crystallisation from acetone they all melted at 75·5–75·8°. The collected material (7·7 g.) was saponified and the resulting alcohol crystallised twice from 4 litres of benzene-acetone (1:2) at 37° when the melting-point was constant at 91·9–92·2°. The yield was 5 g. (Found: C, 82·6; H, 14·2 %. $C_{34}H_{70}O$ requires C, 82·5; H, 14·3 %.) The large colourless rhombic plates obtained from acetone gave an excellent X-ray photograph (Series B, 266); up to 36 orders measuring 74·64 Å. in the B or short form, with no trace of the A or long form. The purity of the alcohol was checked by reduction *via* the iodide to *n*-tetratriacontane [Pollard *et al.*, 1931]. After treat-

ment with sulphuric acid this had M.P. 72·6–72·8°; S.P. 72·5°, and heating and cooling transition temperatures of 69·2–4° and 68·5° respectively, in excellent agreement with those of the pure synthetic paraffin [Piper *et al.*, 1931]. *n*-Tetratriacontanoic acid was prepared by oxidising the alcohol (0·5 g.) with chromium trioxide in glacial acetic acid in the usual way [Pollard *et al.*, 1931]. The crude acid was recrystallised twice from acetone at 37°, when it separated in large colourless rhombic plates, M.P. 98·3–98·5°. It gave an excellent X-ray photograph (Series B 270) in the B or long form measuring 80·4 Å., with no trace of the C or short form.

Oxidation of cocceryl alcohol to the corresponding ketonic acid. 13 g. of cocceryl alcohol dissolved in 3 litres of glacial acetic acid were treated with 7·2 g. of (moist) chromium trioxide at 65–70° for 20 minutes. The resulting green solution was poured with stirring into 7 litres of water and the solid which separated washed thoroughly with water. The salmon-pink powder thus obtained was dissolved in 2 litres of boiling 95 % alcohol, which was filtered hot. To the hot filtrate a further 200 ml. of boiling alcohol containing 10 g. of potassium hydroxide, followed by 300 ml. of boiling alcohol containing 15 g. of calcium chloride, were added. After boiling under reflux for 1 hour the mixture was filtered hot to separate the precipitated calcium soaps. These were extracted three times with 1500 ml. of boiling alcohol to remove any unchanged cocceryl alcohol and then dissolved in 600 ml. of boiling glacial acetic acid to decompose the soaps. The free ketonic acid was precipitated by pouring into excess of water, collected and washed repeatedly with water and cold acetone. It was then crystallised from 1500 ml. of acetone. The yield was 7·5 g. After recrystallisation from acetone at 37° the M.P. was 106·5–107°. (Found: C, 78·1; H, 12·9 %. $C_{34}H_{66}O_3$ requires: C, 78·1; H, 12·7 %.)

Oxime of the ketonic acid. 7 g. of the keto-acid, 7 g. of hydroxylamine hydrochloride and 12 g. of potassium hydroxide in 1500 ml. of 95 % alcohol were boiled under reflux overnight. The cooled solution was poured into 4500 ml. of dilute sulphuric acid. The solid which separated was extracted with 1200 ml. of cold ether, which was afterwards filtered. The ether was removed, and the solid residue crystallised from ice-cold acetone; 5·0 g. of oxime in microscopic needles, M.P. 62·5–63°, were obtained. (Found: C, 76·0; H, 12·6; N, 2·5 %. $C_{34}H_{66}O_2.NOH$ requires: C, 75·9; H, 12·7; N, 2·6 %.)

Transformation of the oxime and hydrolysis of the resulting amides. The procedure followed closely that observed with the oxime of 13-keto-*n*-dotriacontanoic acid. 4·5 g. of oxime gave 2·5 g. of mixed amides which were hydrolysed for 4 hours with concentrated hydrochloric acid in a sealed tube. The appropriate treatment gave ether-alcohol-insoluble material weighing 0·67 g. (Fraction A); a very small amount of benzene-alcohol-insoluble material (Fraction B) which was neglected, alcohol-insoluble soaps (Fraction C) and alcohol-soluble soaps (Fraction D).

Fraction A. This was extracted twice with boiling benzene. On cooling, the benzene deposited 0·28 g. of crystalline material. This was recrystallised from benzene, extracted thoroughly with ether and the residue crystallised from a mixture of 20 ml. of ether and 10 ml. of alcohol. 0·11 g. of *n*-nonadecanamine hydrochloride were obtained. (Found: C, 71·4; H, 13·3; N, 4·2 %. $C_{19}H_{41}N.HCl$ requires: C, 71·3; H, 13·1; N, 4·4 %.) The benzoate, alone and mixed with the synthetic product, melted at 88°.

The material originally insoluble in benzene melted at 157–159°. After recrystallisation first from water and then from 2*N* hydrochloric acid it melted at 166°. The crystalline form and solubilities were similar to those of the

12-aminolauric acid hydrochloride mentioned above, and there was no doubt that it was the expected, but unknown, 14-aminomyristic acid hydrochloride. Again we found the amino-acid very difficult to purify, and after repeated treatment with boiling acetone and boiling benzene there remained only 18 mg. of material melting at 168°. (Found: (micro-Kjeldahl) N, 4·7 %. $C_{14}H_{31}N.HCl$ requires: N, 5·0 %.) 13 mg. were dissolved in hot water and the requisite amount of alkali added. The free acid crystallised on cooling in thin leaflets, M.P. 181–182°. There was insufficient material for further treatment or analysis. In a mixed melt with 12-aminolauric acid (M.P. 183°) the M.P. was depressed to 168–169°.

Fraction C. The soaps were dissolved in acetic acid, which was then diluted with water. The free acids were collected and crystallised from aqueous acetone. The material thus obtained was extracted with light petroleum (B.P. < 40°). The extract on concentration yielded 38 mg. of fatty acid, M.P. 68°. This and the insoluble residue were joined with the similar products isolated from Fraction D.

Fraction D. This was treated in a similar way to Fraction C. The material soluble in cold petroleum (60 mg.), with the added material mentioned above, was recrystallised from alcohol, acetone and finally from light petroleum. It proved to be *n*-eicosanoic acid. (Found: C, 77·2; H, 12·8 %; mol. wt. by titration 308. $C_{20}H_{40}O_2$ requires: C, 76·9; H, 12·9 %; mol. wt. 312.) It melted at 74·0–74·2°, and in a mixed melt with the synthetic acid (M.P. 75–75·2°) the M.P. was 74·6–74·8°.

The residue insoluble in cold petroleum was extracted with boiling water, which was filtered hot. On cooling, a few mg. of a white crystalline solid separated, M.P. 111–112°. It was thought that this acid would be *n*-tridecane-(1:13)-dicarboxylic acid, M.P. 115°, which has been recently synthesised by Chuit [1926]. As purification of the brassylic acid in the preceding case had proved exceptionally troublesome, and as none of the synthetic acid was available for a mixed melt, the small amount of material was boiled under reflux in 5 ml. of methyl alcoholic hydrogen chloride to prepare the dimethyl ester. This melted at 42°, alone and mixed with the synthetic ester (M.P. 43°) kindly supplied by Dr Chuit. There was insufficient material for analysis.

*15-Hydroxy-*n*-tetratriacontanol.* Cocceryl alcohol (1 g.) in boiling amyl alcohol (100 ml.) was reduced with sodium (8 g. in small portions). The product was washed with water and recrystallised twice from acetone (350 ml.) at 37°, when it was obtained in small colourless rhombic plates, M.P. 98·9–99·1°. The yield was 0·7 g. (Found: C, 80·1; H, 13·8 %. $C_{34}H_{70}O_2$ requires: C, 79·9; H, 13·8 %.) The diacetate was prepared with fused sodium acetate and acetic anhydride in the usual way and recrystallised twice from a small volume of acetone at 0°, when it separated in small rosettes of colourless needles. The melting-point was not sharp, the material became molten at 49° and the melt cleared at 50–51°. (Found: C, 76·6; H, 12·4 %. $C_{38}H_{74}O_4$ requires: C, 76·8; H, 12·5 %.) It is fairly readily soluble at room temperature in ether and acetone.

Summary.

Coccerin, the benzene-soluble, alcohol-insoluble wax of the cochineal insect (*Coccus cacti*) has been investigated in some detail.

The cocceryl alcohol of Liebermann is not a dihydric alcohol containing 30 carbon atoms as he suggested but is 15-keto-*n*-tetratriacontanol. His coccerinic acid is a mixture consisting of 13-keto-*n*-dotriacontanoic acid and *n*-triacontanoic acid.

The generic relationship between the ketonic acid and ketonic primary alcohol present in this wax with the secondary alcohol *n*-nonacosan-10-ol present in the wax of apple cuticle is briefly discussed.

Reasons are given for the suggestion that all previous work on the constitution of insect waxes is in need of reinvestigation.

In conclusion we should like to thank Dr S. H. Piper for several X-ray analyses which have materially aided us in the course of this work; Mrs G. M. Robinson for a sample of 11-bromoundecanoic acid; Chuit, Naef and Co., Geneva, for a sample of methyl *n*-tridecane-(1:13)-dicarboxylate; Dr N. K. Adam for samples of 12-aminolauric acid and brassylic acid prepared by the late Dr Le Sueur; Mr A. L. Bacharach of Glaxo, Ltd., for kindly arranging for the extraction of 15 kg. of cochineal with boiling benzene and Dr F. H. Carr of British Drug Houses for a similar extraction of 60 kg. of cochineal.

REFERENCES.

Becker (1931). *Biochem. Z.* **239**, 235.
Chibnall, Piper, Pollard, Smith and Williams (1931). *Biochem. J.* **25**, 2095.
Chuit (1926). *Helv. Chim. Acta*, **9**, 264.
Jeffreys (1899). *Amer. Chem. J.* **22**, 30.
Lapworth (1907). *J. Chem. Soc.* **91**, 1133.
Le Sueur (1915). *J. Chem. Soc.* **107**, 736.
Liebermann (1885). *Ber. deutsch. chem. Ges.* **18**, 1975.
—— and Bergami (1887). *Ber. deutsch. chem. Ges.* **20**, 959.
Malkin (1930). *J. Amer. Chem. Soc.* **52**, 3739.
Piper, Chibnall, Hopkins, Pollard, Smith and Williams (1931). *Biochem. J.* **25**, 2072.
Pollard, Chibnall and Piper (1931). *Biochem. J.* **25**, 2111.
Robinson (1930). *J. Chem. Soc.* 745.
Schulz and Becker (1931). *Biochem. Z.* **235**, 233.

XLIV. THE PROTEINS OF GRASSES.

III. THE CYSTINE CONTENT OF CERTAIN GRASSES AND OTHER PASTURE PLANT PROTEINS.

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A few years ago Robertson [1928] suggested that since the keratin of wool contained such a high proportion of cystine (about 13 %) the carrying capacity of any country for sheep which are grown for wool production might well be determined by the capacity of its pasture plants to produce cystine. Attention thus became focused on the cystine content of grasses and other pasture plants. Now the major part of this cystine will presumably be present as a constituent of the leaf proteins, and to determine this protein-cystine it is necessary to hydrolyse the protein with strong acid or (less satisfactorily) to digest it with appropriate proteolytic enzymes. As no method for preparing these proteins has hitherto been available earlier workers have found it necessary to submit the whole grass or pasturage itself to acid hydrolysis or to enzymic digestion. This means, in effect, that the proteins in the leaf material have been treated in the presence of from three to six times their own weight of cellulose, sugars, organic acids *etc.*, and it is not at all surprising that under these very unsatisfactory conditions the highly specific colour reaction of Sullivan for cystine was found difficult to carry out and indicated the presence of only a trace of this amino-acid [Aitken, 1930; Evans, 1931; Henrici, 1932]. Evans [1931] also, assuming the alkali-labile sulphur to represent two-thirds of the cystine-sulphur, found that the various samples of pasturage examined by him might contain from 0·07 to 0·18 % of cystine on the basis of dry matter. He pointed out however that not all the labile sulphur of proteins can correctly be ascribed to cystine and suggested that the true cystine content of grass must be less than 0·1 % of the dry matter.

Rimington and Bekker [1932], adopting the figure 0·01 % of dry matter as a fair average value for the cystine content of grass, calculate that a sheep producing a 12 lb. fleece, which contains about 0·78 lb. of cystine, would have to eat 21·4 lbs. of dry grass daily in order to obtain this amount of preformed cystine. Since sheep under optimum conditions will consume only 4 lbs. of dry matter daily they conclude that grasses are deficient in cystine. Woodman and Evans [1932] disagree with this conclusion, and point out that Evans's value, 0·1 % of the dry matter, for the cystine content of cultivated English pasturage would enable sheep to secure the cystine necessary for a 12 lb. fleece during a grazing period of only 195 days. Rimington *et al.* [1933] maintain, however, that the value of 0·1 % for the cystine is too high, and in support of their contention instance a stud ram which produced a fleece of 46 lbs. on a diet consisting almost entirely of lucerne.

These discussions were based on inadequate experimental evidence. During the past three years methods have been perfected in this laboratory for pre-

paring the proteins of grasses and other forage plants, and we have now completed a fairly searching enquiry into their cystine content. We first determined cystine by the well-known methods of Sullivan. The protein hydrolysates were, however, deeply coloured and could not be satisfactorily decolorised by means of norite or kaolin without large losses of cystine. Our results with cocksfoot protein, which we used exclusively in our preliminary experiments, suggested only 0·1–0·2 % of cystine.

The method of Vickery and White [1933], however, details of which were kindly supplied to us some months before publication by Dr Vickery, gave very much greater values, 1·1–1·2 %. According to this method the protein is hydrolysed in the presence of tin, which reduces the cystine to cysteine as it is set free. The cysteine is then precipitated from solution under appropriate conditions as the very insoluble cuprous mercaptide. This precipitate is decomposed with hydrogen sulphide, the cuprous sulphide removed and the organic sulphur in the clarified solution determined. In all the cases examined by them the cystine content of the protein calculated from this sulphur value agreed reasonably well with the content determined by Sullivan's procedure. In the case of our leaf proteins, as mentioned above, there was a great discrepancy. Moreover the value for cystine determined by Sullivan's method on the clear filtrate from the cuprous sulphide was similar to that obtained on direct hydrolysis. Clearly the Vickery and White procedure was estimating, in addition to the sulphur of cystine, the sulphur of (1) an unknown amino-acid giving an insoluble cuprous salt, or (2) products arising from the decomposition of cystine during the acid hydrolysis of the protein in the presence of the small amount of impurity which all our grass protein preparations contain.

We have been able to eliminate the latter alternative by making use of the new modification of Sullivan's method introduced by Prunty [1933]. The cystine in the hydrolysate is reduced to cysteine by means of zinc in acid solution instead of with sodium cyanide, and during the reduction the hydrolysate is almost completely decolorised. Accurate and easily reproducible values for the cystine content of the leaf proteins were readily obtained. Added cystine could also be recovered in 90–95 % yield, showing that no appreciable destruction of cystine was taking place during hydrolysis. Additional proof was afforded by experiments in which the protein was hydrolysed by enzymes.

One very interesting result of our research has therefore been the clear proof that leaf proteins must contain at least one unknown sulphur-containing amino-acid, because Vickery and White have shown that methionine, which is present in these proteins, is not precipitated under their conditions as a cuprous salt. This unknown amino-acid yields part of its sulphur as sulphide on alkaline hydrolysis and may quite likely be the same product which the experiments of Zahnd and Clarke [1933] show to be present in egg-white.

The cystine content of the pasture leaf proteins, determined by the Prunty-Sullivan colorimetric method, varies between 0·25 and 1·25 %, as shown in Table I, and by calculation it is possible to show quite definitely that a mixed herbage of the type which constitutes the pasturage of sheep in this country contains an adequate amount of cystine for the production of a 12 lb. fleece. Lucerne protein has also been examined, and as it is relatively rich in cystine there is no reason to doubt that the prize ram referred to by Rimington *et al.* [1933] also obtained all the necessary cystine from its diet. As the sulphur of the unknown amino-acid referred to above is not stable to alkali it is clear that Evans's determinations of labile sulphur in English pasture grasses were not a valid basis for calculating their cystine contents.

Experimental.

Preparation of the proteins. Full details have been given in a previous paper [Chibnall *et al.*, 1933]. The nitrogen content of the various preparations ranged from 10 to 15 %, owing to the presence of non-nitrogenous impurities whose chemical composition is not yet clearly understood [Miller and Chibnall, 1932]. The percentage of cystine determined on these preparations therefore falls short of the percentage in the protein by an amount depending on the proportion of these impurities present. Leaf proteins known to be pure contain from 15·7 to 16·7 % N [Chibnall and Grover, 1926]. On the assumption that the grass and other pasture plant proteins should also contain 16 % N we have, in the Tables given below, calculated the percentage of cystine in the protein on this basis. Adoption of this figure has the additional advantage that in our subsequent discussion on the cystine content of pasture in relation to wool production we are able to make direct comparison with the conventional protein (N × 6·25) of the agricultural chemist.

Colorimetric estimation of cystine by the Prunty modification of Sullivan's method. The protein (0·5–1·0 g.) was heated with 25 ml. of 20 % hydrochloric acid in a flask immersed in a boiling water-bath for 2 hours, and then gently boiled under reflux overnight. The flask was cooled in ice water, 40 % aqueous sodium hydroxide added to p_H 2·5 and the precipitated humin separated from the yellow or orange solution by centrifuging. To the humin remaining in the centrifuge-tube 5–10 ml. of dilute sodium chloride solution previously adjusted to p_H 2·5 were added, and the tube was immersed in a boiling water-bath for 10 minutes, the mixture being frequently stirred. The humin was again centrifuged off and the washing repeated at least eight times in all. Further washings gave no appreciable amount of cystine by the colorimetric method. The use of aqueous sodium chloride instead of water for washing the humin was necessary to prevent the formation of colloidal suspensions. The final solution was brought to a convenient volume, usually 100 ml., the last few washings being concentrated, if necessary, *in vacuo*.

Two portions of 5 ml. were measured into centrifuge-tubes, and to each was added 1 drop of concentrated hydrochloric acid and a few mg. of zinc dust. The tubes were then heated in a boiling water-bath for 5 minutes, cooled and centrifuged. The faintly yellow solutions were decanted into 15 ml. standard flasks, containing 1 ml. of 25 % aqueous sodium hydroxide and 2 ml. of 10 % aqueous sodium cyanide, the tubes being washed out with the requisite amount of water to make up the standard volume. At the same time a convenient amount of cystine was reduced and treated in a similar way. To one of the unknown solutions (A) and to the known cystine solution (S) were added 1 ml. of a 1 % solution of sodium β-naphthoquinonesulphonate and then immediately 5 ml. of a 10 % solution of sodium sulphite in 0·5N aqueous sodium hydroxide; while to the other unknown (B) was added only the sulphite solution. After remaining at 25° for 30 minutes to each of the three solutions was added 1 ml. of a 2 % solution of sodium hydrosulphite in 0·5N aqueous sodium hydroxide. The colour of a known depth of solution (A) was then compared with the colour of the same depth of the blank solution (B), superimposed on a variable depth of the standard solution (S), using a Klett colorimeter. No rapid fading of the red colour has been observed with our hydrolysates such as that recorded by Prunty in the case of gelatin. After standing in the air for half an hour the solutions become slightly darker, but the addition of a very small amount of sodium hydrosulphite at once restores the original colour. The results for the pasture leaf proteins are fully set out in Table I.

Table I. *The cystine content of pasture leaf proteins estimated by the Prunty modification of Sullivan's method.*

Species	Sample of leaf protein: Total N (ash-free) %	Sample of leaf protein: Cystine %	True protein (N = 16 %) Cystine %
Red clover (*Trifolium pratense*)	12·6	0·16	0·21
Bent (*Agrostis* sp.)	10·3	0·15	0·24
Cocksfoot (*Dactylis glomerata*) (AB)	14·6	0·26	0·28
,, (AC)	13·4	0·20	0·24
,, (AD)	13·2	0·20	0·24
,, (AG)	13·2	0·21	0·26
,, (AH)	13·5	0·23	0·27
Rough-stalked meadow grass (*Poa trivialis*)	13·8	0·23	0·27
Wild white clover (*Trifolium repens*)	13·2	0·26	0·32
Timothy (*Phleum pratense*)	13·8	0·29	0·34
Perennial ryegrass (*Lolium perenne*)	12·7	0·38	0·48
Yorkshire fog (*Holcus lanatus*)	11·8	0·36	0·49
Italian rye-grass (*Lolium italicum*)	14·0	0·59	0·67
Tall fescue (*Festuca elatior*)	13·6	0·60	0·70
Lucerne (*Medicago sativa*) (A)	14·4	0·66	0·73
Crested dog's tail (*Cynosurus cristatus*)	14·9	0·70	0·75
Meadow fescue (*Festuca pratensis*)	13·9	0·67	0·80
Red fescue (*Festuca rubra*)	14·2	0·77	0·87
Hard fescue (*Festuca duriuscula*)	14·6	0·85	0·90
Chewings fescue (*Festuca rubra* var. *Fallax* (Hack.))	14·0	0·85	0·93
Lucerne (*Medicago sativa*) (B)	15·8	1·20	1·20

Possible losses in the colorimetric estimation of cystine. The possibility that the impurities present in our protein preparations were causing extensive breakdown of cystine was investigated by adding a known amount of this amino-acid before hydrolysis. The results of two typical experiments with cocksfoot and crested dog's tail proteins are given in Table II, and show a recovery of added

Table II. *Showing the recovery of added cystine.*

Protein preparation	Cystine %	Cystine added %	Total cystine estimated %	Recovery of added cystine %
Standard HCl hydrolysis (Prunty):				
Cocksfoot (AC)	0·19	0·15	0·31	91
,,	0·19	0·50	0·65	94
Crested dog's tail	0·70	0·70	1·36	97
,,	0·70	0·70	1·34	95
Hydrolysed with H_2SO_4 in presence of tin:				
Cocksfoot (AB)	0·25	—	0·21	84
,,	0·25	0·63	0·74	84

cystine in each case of over 90 %. Lugg [1933] has shown that cysteine, produced from cystine by heating with stannous chloride and hydrochloric acid, may suffer almost complete decomposition if hydrolysed in the presence of relatively large amounts of sugars and amino-acids. With pure proteins Vickery and White [1933] experienced no such loss. As hydrolysis with hydrochloric acid in the absence of oxygen [Okuda, 1926] showed that cocksfoot protein contained about 0·1 % of cysteine we think it worth while to record the following experiment.

Two portions of 1 g. of cocksfoot protein (AB) were taken, and to one were added 6·3 mg. of cystine. Each portion was then hydrolysed with 20 ml. of 8 *N* H_2SO_4 for 20 hours in the presence of 1 g. of granulated tin. The solutions were

cooled, 40 % aqueous sodium hydroxide was added to p_H 2·5 and the mixtures, each containing the humin in suspension, were saturated with hydrogen sulphide. The resulting pale yellow solutions were cleared by centrifuging, and the residue of stannous sulphide and humin was washed eight times successively with dilute hydrochloric acid. In neither case did the last two washings give any nitroprusside reaction. The solutions were concentrated *in vacuo* and the cystine+cysteine present estimated colorimetrically in the usual way. The results are given in Table II. If it be assumed that the cystine content of the protein be that given on standard hydrochloric acid hydrolysis, then the amount of cystine estimated in each case amounted to 84 %. The loss is possibly due in part to the fact that air oxidation of cysteine to cystine (which must have taken place to a small extent during the concentration *in vacuo*) is not strictly quantitative, but we think that the major loss is due to adsorption of cysteine on the stannous sulphide; even greater losses (20–30 %) were found by Lugg [1933] when cystine was heated with stannous chloride and hydrochloric acid, the metal being subsequently removed as sulphide. The results show fairly conclusively that Prunty's modification of the Sullivan method estimates 90–95 % of the total cystine and cysteine present in the pasture leaf proteins.

The alkali-labile sulphur as a measure of cystine content. The procedure employed by Osborne [1902] was followed. The results were somewhat erratic, as Osborne found with seed proteins, but representative values for the alkali-labile sulphur of cocksfoot protein are given in Table III. If we accept Osborne's conclusion that this represents about two-thirds of the total cystine-sulphur, the value for cystine in each case would be approximately three times that given by the Prunty-Sullivan method. It appears from this result that a determination of alkali-labile sulphur estimates not only the sulphur of cystine but also in large part the sulphur of the unknown sulphur-containing amino-acid whose presence in the hydrolysis products of these leaf proteins is clearly demonstrated by the Vickery and White procedure outlined below. It follows therefore that the cystine values for pasture grasses quoted by Woodman and Evans [1932], which are based on determinations of alkali-labile sulphur by Evans [1931], are not valid.

Table III. *Showing the distribution of organic sulphur in certain pasture leaf proteins.*

		True protein (N = 16 %)				
Species	Protein preparation Total N (ash-free) %	Cystine-sulphur (Sp) (Prunty-Sullivan) %	Cystine-sulphur (Sv) (Vickery and White) %	Sv − Sp	Alkali-labile sulphur %	Total sulphur %
Cocksfoot (AB)	14·6	0·072	0·26	0·19	0·12	1·10
,, (AC)	13·4	0·061	0·31	0·25	0·15	1·30
,, (AG)	13·2	0·063	0·31	0·25	—	1·40
Tall fescue	13·6	0·184	0·29	0·11	—	1·21
Red fescue	14·2	0·221	0·42	0·20	—	1·56
Hard fescue	14·6	0·24	0·38	0·14	—	1·85
Perennial ryegrass	12·7	0·125	0·26	0·14	—	1·56
Lucerne	14·4	0·19	0·28	0·09	—	1·55

Estimation of cystine(mercaptide)-sulphur by the method of Vickery and White [1933]. 5–10 g. of protein were hydrolysed in the presence of tin and the cysteine precipitated as the cuprous mercaptide. The procedure of these authors gave

consistent results without difficulty, and, as will be seen from Table III, the values for cystine(mercaptide)-sulphur are in all cases greatly in excess of those obtained by the Prunty-Sullivan colorimetric method. If the latter represent the true cystine-sulphur, as we believe, then it follows that the cuprous oxide must have precipitated at least one other sulphur-containing amino-acid. Confirmation of the low cystine content of the cuprous precipitate was obtained in the following experiment.

46 g. of hard fescue protein, containing 0·84 % of cystine by the Prunty-Sullivan colorimetric method, were treated according to the procedure of Vickery and White. The cuprous mercaptide was then decomposed with hydrogen sulphide in the presence of sulphuric (not hydrochloric) acid. The cuprous sulphide was centrifuged off and washed repeatedly by boiling with dilute sulphuric acid until the washings gave no nitroprusside reaction for cysteine. The clean solution and washings were united, adjusted to p_H 7 with barium hydroxide and aerated for 1 hour, after which time no free cysteine could be detected by the nitroprusside reaction. The solution was then made slightly acid with sulphuric acid and concentrated *in vacuo*. The precipitate of barium sulphate was centrifuged off and washed repeatedly with 0·002 *N* sulphuric acid. The solution and washings were united and concentrated *in vacuo* to 250 ml. Cystine, by the Prunty-Sullivan method, amounted to 0·27 g., equivalent to 0·6 % of the protein taken for analysis. Vickery and White show that a certain amount of cysteine destruction occurs during the decomposition of the cuprous mercaptide. This amount of cystine contains 0·072 g. of sulphur, whereas the total organic sulphur present was 0·173 g.

The solution was then concentrated *in vacuo* to 25 ml. and an equal volume of alcohol added. The pale brown precipitate which came out in the course of a few days was filtered off, dissolved in a few ml. of dilute hydrochloric acid and a small residue of barium sulphate filtered off. The clear filtrate was then cooled to 0° and saturated with hydrogen chloride gas. The mass of fine needle-shaped crystals, typical of cystine dihydrochloride, which separated overnight, was filtered off and washed with a little cold absolute alcohol. The weight was 0·14 g., and the mother-liquor on treatment gave a further 0·06 g., making 0·22 g. in all, a yield of 60 % of that estimated colorimetrically. For analysis (Schoeller) the material was dried at 60° *in vacuo* over phosphorus pentoxide. (Found: S, 20·6, 20·4 %. $C_6H_{14}O_4N_2S_2Cl_2$ requires: S, 20·45 %.) The yield of cystine compares favourably with that of Vickery and White, if it be remembered that our solutions contained nearly an equal amount of another sulphur-containing amino-acid.

The liberation of cystine during enzymic digestion. Additional evidence that the Prunty procedure gives a true estimate of the cystine content of the proteins, and that the Vickery and White procedure is not estimating any decomposition products of cystine, was obtained from experiments in which the protein was broken down by enzymic digestion and was therefore not subject to the action of products set free from the non-protein impurities by the strong acid hydrolysis. Six digests of cocksfoot protein and one of Italian ryegrass protein have been examined; we shall however only quote one result of the former. Details of the experiments follow closely those concerned with the amide-N of proteins [Damodaran, 1932, Damodaran *et al.*, 1932], the enzymes used being pepsin, trypsin and intestinal (pig) erepsin in succession.

The following account concerns the digestion of cocksfoot protein (AB). (Data corrected for added enzyme preparation.) The protein (9·5 g.) was suspended in 400 ml. of dilute hydrochloric acid (p_H 1·9) containing 0·4 g. of pepsin.

The protein rapidly settled, and the flask was gently shaken at intervals during 41 hours. At the end of that time much of the protein still remained undissolved, the supernatant liquid containing only 16·6 % of the total N. Previous digests had shown that no appreciable amount of cystine was liberated by pepsin [*cf.* Jones and Gersdorff, 1933]. *N* sodium hydroxide solution was then added to p_H 8·2, when the major part of the protein residue was at once dissolved. 0·4 g. of trypsin was next added, together with 0·5 % of tricresol as antiseptic. It was necessary to adjust the digest to p_H 8·2 every few hours. Cystine and amino-N were determined at intervals, as shown in Table IV. At the end of 72 hours' digestion, when no appreciable protein residue remained, 40 ml. of pig erepsin were added. The solution was maintained at p_H 8·0, and the amino-N reached a maximum at the end of a further 17 hours. In the case of cocksfoot no appreciable increase of cystine was observed with erepsin, though a definite increase was noted in the case of Italian ryegrass. All the enzymic digests were very deeply coloured, and on neutralisation to about p_H 2·5 a large amount of material, partly humin and partly of a proteose nature, was thrown down and was difficult to wash. The cystine determinations were therefore not as consistent or as reliable as those obtained with corresponding acid hydrolysates. It was observed in each case that if the digestion with either trypsin or erepsin were prolonged the cystine value fell slowly from the maximum, indicating a slow secondary decomposition of cystine under the conditions of the experiments.

The results however leave no doubt that the amount of free cystine liberated was of the magnitude that one would expect if the total cystine in the protein were that given by the Prunty-Sullivan colorimetric method and not that given by the Vickery and White mercaptide method; in the case of the cocksfoot protein quoted in Table IV the liberation of cystine was 70 % and of amino-N was 58 % of the maximum given on acid hydrolysis, while with the Italian ryegrass the corresponding figures were 62 % and 64 % respectively.

Table IV. *Showing the amount of cystine set free during the enzymic digestion of cocksfoot and Italian ryegrass proteins.*

		Cocksfoot protein (AB) 14·6 % N 0·25 % cystine		Italian ryegrass protein 14·0 % N 0·58 % cystine	
Enzyme	Duration of digestion (hours)	Cystine (% total protein)	Amino-N (% total N)	Cystine (% total protein)	Amino-N (% total protein N)
Pepsin	41	—	2·4	—	—
Trypsin	24	0·09	—	0·20	—
,,	48	0·10	—	0·24	—
,,	72	0·18	28	0·30	34·8
Erepsin	17	0·17	40·2	0·36	44·8
,,	41	0·15	40·2	0·33	45·0

The total organic sulphur of the proteins. The experimental data given above show that the hydrolysis products of all the pasture leaf proteins examined contain cystine, and that this can be readily estimated by the highly specific colorimetric method of Sullivan. They show also that another amino-acid, probably one containing a sulphydryl group, must also be present. It will be seen from Table III, however, that these two amino-acids, both of which are estimated by the method of Vickery and White, account for only about one-quarter of the organic sulphur present in the proteins. An estimation of methionine on cocksfoot protein by the indirect method of Baernstein [1932]

suggests that the remainder may be due to this amino-acid. Further investigations into the constitution of the unknown amino-acid and of the methionine content of the proteins are in progress.

Reference to Table II shows that in the case of cocksfoot protein with a low cystine content the Prunty-Sullivan method estimates only 90–95 % of the total amount present. If the protein preparation were very impure, such as that of bent or yarrow (N = 10 %), it is probable that the loss could be even greater. When preparing the data for Table V, therefore, we have in these cases made what we consider a legitimate allowance for a possible loss during the estimation of from 5–15 % of the total cystine. We have in a few instances determined the cystine content of the dried plant material itself by the Prunty-Sullivan procedure described above. After the reduction process the solutions are somewhat darker than in the case of the proteins, but the red colour subsequently developed by the cysteine can be readily estimated. Low values were always obtained, and added cystine was destroyed to the extent of 40–50 %. This result was not unexpected.

The cystine content of pastures and wool production. Table V gives the calculated amount of cystine, in terms of total dry matter, present in the various

Table V. *Cystine content of pasture plants at various protein levels.*

Species	Percentage of cystine in protein (N = 16 %)	Cystine as a percentage of total dry matter in the herbage, containing Protein 8 %	Protein 12 %	Protein 16 %	Protein 20 %	Protein 24 %	Protein 28 %
Yarrow, red clover	0·25	0·02	0·03	0·04	0·05	0·06	0·07
Bent, cocksfoot, rough stalked meadow grass	0·30	0·024	0·036	0·048	0·06	0·072	0·084
Timothy, wild white clover	0·35	0·028	0·042	0·056	0·07	0·084	0·098
Perennial ryegrass, Yorkshire fog	0·50	0·04	0·06	0·08	0·10	0·12	0·14
Italian ryegrass, tall fescue	0·70	0·056	0·084	0·112	0·14	0·168	0·196
Lucerne (A)	0·75	0·06	0·09	0·12	0·15	0·18	0·21
Crested dog's tail, meadow fescue	0·80	0·064	0·096	0·128	0·16	0·192	0·224
Red fescue, hard fescue	0·90	0·072	0·108	0·144	0·18	0·216	0·252
Chewings fescue	0·95	0·076	0·114	0·152	0·19	0·228	0·266
Lucerne (B)	1·2	0·096	0·144	0·192	0·24	0·288	0·336

pasture plants at certain definite protein levels. These values are based on the assumption that the protein is the sole source of cystine. They must therefore be regarded only as minimum values, because cystine or cystine peptides may possibly be present in the water-soluble, non-protein products of the plants, and these have not yet been investigated.

Before attempting to assess the value of these plants as diet for sheep it is necessary to make one or two observations concerning the actual samples which were used for preparing the proteins. All the grasses were taken from plots which had been cut at least once a fortnight, and the samples consisted almost entirely of blades. The red clover and lucerne (A) were each cut about 1 inch above ground, and both samples consisted of somewhat stunted plants with relatively few leaflets. The protein preparations therefore consist of leaf protein well adulterated with that from the stems. The wild white clover and lucerne (B),

on the contrary, were from luxuriant crops, and both were cut with a scythe held well above the ground so that the sample consisted chiefly of leaflets obtained from the upper few inches of the plants. The protein preparations, therefore, are very rich in the leaf protein. This accounts for the striking difference between the two samples of lucerne protein. Sample A, which contained 14·4 % N and 0·73 % cystine, was prepared from lucerne containing 17·2 % true protein. Sample B, which contained 15·8 % N and 1·2 % cystine, was prepared from lucerne grown in Connecticut, U.S.A., containing about 28 % true protein [Chibnall and Nolan, 1924].

With regard to the cystine content of pastures and wool production the data given in Table V can only be discussed in general terms because in actual practice the herbage is always mixed and may change considerably in botanical composition during the season. Furthermore, the question of the amount of ingested cystine which is catabolised or excreted is outside the scope of this paper, so that in the following discussion we can do no more than attempt to show that the minimum amount of cystine ingested is greater than that required for wool-protein synthesis.

If we assume that a sheep eats pasturage containing 4 lbs. of dry matter daily, then the 0·39 lb. of cystine necessary for an average British fleece of 6 lbs. would be secured during one year on a diet containing only 0·0265 % of cystine on the basis of dry matter. This is well below the amount which would be provided by the poorest of pasture consisting chiefly of bent, even if we ignore the fact that such pastures would generally contain a small proportion of one of the fescues, which are rich in cystine. The winter grazing (8 % protein) might be on the borderline, but for 6–8 months during the growth period the protein level would exceed 12 %, and the cystine content of the diet would be in excess of 0·04 %. A 12 lb. fleece, which is the weight discussed by Rimington and Bekker [1932], contains 0·78 lb. of cystine, and for this intake the sheep would require a diet throughout the year containing 0·053 % of cystine on the basis of dry matter. In Great Britain a cultivated pasture of the type which might be expected to carry sheep bearing such a fleece would probably consist in large part of perennial ryegrass, with subordinate amounts of Italian ryegrass, rough-stalked meadow grass, wild white clover and bent at the appropriate season. From March to October the pasture would contain on an average nearly 20 % of protein on a basis of dry matter, so that the cystine content of the diet throughout this period would vary between about 0·08 and 0·1 %. This grazing period alone therefore would provide more than enough cystine for the fleece. During the winter months the protein content of the grazing would fall, but the early appearance of Italian ryegrass would help materially in maintaining an average cystine content in the diet of about 0·05–0·06 %. From an agricultural point of view these descriptions of poor and good quality pastures are necessarily very incomplete, and they are used here only as simplified examples to illustrate our general contention that the pastures of Great Britain almost certainly contain considerably more cystine than the sheep, which they normally carry, require for wool-protein synthesis, especially if it be remembered that sheep will always eat preferentially the young tips of the leaves, which are the richest in protein and therefore in cystine.

In many countries lucerne meal forms one of the principal winter rations of sheep. This has a protein content of about 15 % and is made from cuttings of the whole plant, corresponding to lucerne (A) in Table V. It would therefore have a cystine content of about 0·12 %. If it contained proportionately more leaf material than our sample, which is very probable, then the cystine content

at this protein level would approach the value 0·19 % for lucerne (B) in Table V. These figures are very high indeed when compared with the minimum intake required for the 6 and 12 lb. fleeces mentioned above.

The South African stud ram referred to by Rimington *et al.* [1933], which clipped a record fleece of 46 lbs., was stated to have been fed almost entirely on lucerne. Such a fleece would contain about 3 lbs. of cystine, and on a diet containing 4 lbs. of dry matter daily this amount of cystine would be consumed in one year if the lucerne contained 0·21 % of cystine. This is higher than the value one would expect from ordinary lucerne meal, but a prize stud animal of this type would undoubtedly be given the best possible diet. During the appropriate season it would be turned out to feed on fresh lucerne, and it would undoubtedly choose in preference the leafy lucerne tops. One of us (A. C. C.) analysed many samples of such material collected during the summer of 1924 from crops growing around New Haven, Connecticut, U.S.A., and the true protein content in all cases was never less than 24 % and generally 30 % of the total dry weight. Such material would contain from 0·25–0·3 % of cystine. Lucerne leaf-meal, containing 20–24 % of protein, and hence 0·24–0·27 % cystine, might have formed part of the diet in the stall. In the absence of precise data on the ram's diet, therefore, a cystine intake more than sufficient even for a 46 lb. fleece does not appear extravagant.

Summary.

The cystine content of several typical grass and other pasture plant proteins has been determined by Prunty's modification of Sullivan's colorimetric method. The grass proteins all contain cystine, in amount varying from 0·3 to 0·95 %. Lucerne leaf protein is particularly rich in cystine and contains 1·2 %.

The gravimetric method of Vickery and White gives in all cases a much higher value for cystine-sulphur than the amount shown to be present and must include the sulphur of an unknown amino-acid.

The protein-cystine content of the grasses and other pasture plants at various protein levels has been calculated, and it appears that pasturage contains ample cystine for the wool-protein requirements of the sheep which it normally carries.

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REFERENCES.

Aitken (1930). *Biochem. J.* **24**, 250.
Baernstein (1932). *J. Biol. Chem.* **97**, 663.
Chibnall and Grover (1926). *Biochem. J.* **20**, 108.
—— Miller, Hall and Westall (1933). *Biochem. J.* **27**, 1879.
—— and Nolan (1924). *J. Biol. Chem.* **62**, 173.
Damodaran (1932). *Biochem. J.* **26**, 235.
—— Jaaback and Chibnall (1932). *Biochem. J.* **26**, 1704.
Evans (1931). *J. Agric. Sci.* **21**, 806.
Henrici (1932). *Eighteenth Report, Dir. of Vet. Services and Animal Ind.* Pretoria, August 1932, p. 579.
Jones and Gersdorff (1933). *J. Biol. Chem.* **101**, 657.

Lugg (1933). *Biochem. J.* **27**, 1022.
Miller and Chibnall (1932). *Biochem. J.* **26**, 392.
Okuda (1926). *Proc. Imp. Acad. Tokyo*, **2**, 277.
Osborne (1902). *J. Amer. Chem. Soc.* **24**, 140.
Prunty (1933). *Biochem. J.* **27**, 387.
Rimington and Bekker (1932). *Nature*, **129**, 687.
—— —— and Kellermann (1933). *Nature*, **132**, 63.
Robertson (1928). *Coun. Sci. Ind. Res. Australia, Bull.* No. **39**.
Vickery and White (1933). *J. Biol. Chem.* **99**, 701.
Woodman and Evans (1932). *Nature*, **130**, 1001.
Zahnd and Clarke (1933). *J. Biol. Chem.* **102**, 171.

XLV. THE PENETRATION OF ERYTHROCYTES BY ANIONS.

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THE unequal penetration of ions into erythrocytes has been the subject of numerous observations. Thus the mature cell although impermeable to cations, contains large quantities of potassium, and this in spite of its being suspended in plasma which contains little potassium and much sodium. Again, though erythrocytes are permeable to anions the latter show varying degrees of penetration, and even anions which permeate freely, frequently show an anomalous distribution; the concentration of normal cell-bicarbonate is higher than would have been anticipated from a simple Donnan equilibrium, while Hastings and Van Dyke [1931] have shown that a similar excess of bromide occurs, if bromide is added to the fluid in which erythrocytes have been suspended.

The present investigation is concerned with the entry into erythrocytes of various acids or anions and attempts to correlate certain physical properties with their ability to penetrate the cell membrane.

The relative permeation of anions may be determined from the amount of one anion entering the cell in a given period of time compared with the amount of a second anion entering during the same period. Alternatively, one can expose erythrocytes to a solution containing two salts and estimate their relative concentrations in the cells. The second method has been adopted chiefly because differences in anion permeation, which are barely perceptible with cells suspended in solutions of single salts, are greatly magnified in solutions of the mixed salts, the more penetrating ion greatly depressing the entry of the less penetrating ion. Chloride was adopted as the standard anion and the second anion B was estimated indirectly and sometimes both directly and indirectly. The indirect method is based on the following assumptions:

(1) *If erythrocytes are suspended in KCl solutions, chloride diffuses across the cell membrane and equilibrium is reached within five minutes at 23°.* The validity of this assumption is shown by the figures in Table I.

Table I.

	External p_H 5·5 Ext. Cl 205 m.eq.			Ext. p_H 9 Ext. Cl 88 m.eq.	
Time	% of original volume (V)	Cl (m.eq.)	Time	(V)	Cl (m.eq.)
0 min.	100	50	0	100	50
1 „	94	141	1	140	22
5 „	102	165	5	122	20
60 „	102	170	60	122	20

(2) *The amount of chloride entering erythrocytes from pure KCl solutions depends only on* p_H. This statement is approximate. The erythrocyte contains

haemoglobin and potassium which are unable to penetrate the cell membrane, and according to the Donnan equation, if the external Cl falls, some decrease of cell-Cl must also occur. The actual decrease of Cl however is barely detectable by ordinary analytical methods, and if at the end of an experiment unit volume of cells has changed to V and the final content of Cl is x, then the total Cl taken up by unit volume of cells, Vx, remains practically constant and is independent of the external concentration (Table II). When the external concentration of chloride falls very low, lysis of the erythrocyte occurs, but if the external osmotic pressure is sustained by the addition of glucose, lysis is prevented and it will be seen that the chloride concentration of the erythrocytes is fairly well maintained.

Table II.

(5 minutes' suspension at 23°.)

Ext. Cl	p_H	V	Cl	Vx	p_H	V	Cl	Vx
205	7	88	105	92	5·1	108	182	196
175	7	99	92	91	5·1	129	160	205
146	7	108	79	85	5·1	133	150	200
124	7	124	70	87	5·1	155	125	194
88	7	150	59	88	5·1	—	—	—
22*	—	—	—	—	5·1	88	176	155

* *Plus* glucose 9 %.

If the slowly penetrating glucose is replaced by a slowly penetrating anion like phosphate, the phosphate acts in the same way as the glucose; very little cell-base combines with phosphate, the bulk of it existing as chloride.

(3) *When erythrocytes are placed in a mixture of salt solutions the sum of all the anions entering the cell in five minutes is the same as the amount of chloride entering erythrocytes suspended for the same time in pure KCl solution of the same* p_H. This follows from the preceding observation. If the second anion B permeates more slowly than Cl, Cl enters to excess to satisfy the combining power of cell-base, but with the passage of time, more and more of B diffuses into the cell displacing chloride until true equilibrium is reached. Similarly, if B permeates the more readily, excess of B enters the cell and is subsequently displaced by Cl. In other words the anion distribution during a quasi-stationary state is used as an index of penetrating power.

If therefore at a given p_H, unit volume of cells takes up X m.eq. of Cl from pure KCl solution and if from a solution containing equivalent amounts of KCl and KB, cells take up Y m.eq. of Cl, then the cell content of B, Z m.eq., should equal $X - Y$ m.eq. and the concentration of B relative to Cl, R, may be expressed as Z/Y or $(X - Y)/Y$.

The preceding paragraph itself involves two assumptions:

(*a*) that at a given external p_H, a constant difference exists between the internal and external p_H, whatever the nature of the external salts may be;

(*b*) that at a given p_H inside the erythrocyte, the cell has the power of taking up constant amounts of anions whatever the nature of the diffusing salts.

Neither of these assumptions is strictly correct, but with regard to the second the following may be said. The rate of entry of a substance into a cell depends on its ability to penetrate the cell membrane and on the amount of the substance that the cell can take up. If B is an acid it can exist inside the cell as an anion, as an undissociated acid or as a complex combination with haemoglobin. It follows that if B has a low dissociation constant, or if it forms complexes with haemoglobin comparable with the formation of carbhaemoglobin by CO_2, then its concentration gradient will be relatively greater than that of Cl and it will tend to enter the erythrocyte in greater

amounts; but its power of penetrating the cell membrane will not necessarily be greater than that of Cl.

Now X equals the total anion capacity of cell-base expressed in terms of chloride equivalents; Y represents the amount of Cl combined with cell-base in the presence of anion B and $X-Y$ represents the amount of B combined with cell base also expressed in terms of chloride equivalents. Under these circumstances, it is thought that the value $(X-Y)/Y$ more truly indicates the relative permeation of B and Cl than Z/Y, for Z represents cell-B, dissociated, undissociated and complexly combined.

Further with regard to both the assumptions (a) and (b) it may be said that these approximate statements receive justification from Table III, where, with the exception of the phosphate-chloride system, total cell-anion (B and Cl) is nearly constant at each p_H level.

Table III.

(5 minutes' suspension at 23°.)
Ext. B = 87·5 m.eq.
Ext. Cl = 87·5 m.eq.

Ext. anion (B)	p_H	B + Cl m.eq.	p_H	B + Cl m.eq.	p_H	B + Cl m.eq.
Chloride	7	92	6	150	5·1	205
Iodide	7	98	6	159	5·1	211
Phosphate	7	87	6	133	5·1	182
Iodoacetate	7	92	6	160	5·1	212
Oxalate	7	86	6	146	5·1	198
Salicylate	7	96	6	149	5·1	—

It is thought that the low figure provided by the phosphate-chloride mixtures in acid ranges is due to some specific action of the anion on the cell membrane. Such decrease of permeation also invalidates assumption 3 and may be suspected where increased acidity causes a decrease of cell volume accompanied by agglutination.

It must be clearly understood that though the value $R=(X-Y)/Y$ approximately indicates the relative concentrations of B + Cl in erythrocytes it would only indicate the relative permeation of B + Cl if the cells were naturally chloride-free. But, in fact, R does not show the relative permeation of B and Cl because at the inception of any experiment erythrocytes have a natural chloride content of about 50 m.eq., and chloride therefore has an advantage over B, which is normally absent from the cell. If one were examining a system at equilibrium this initial Cl content would be unimportant, but since the comparison of anion permeation depends on the relative anion concentration before equilibrium has been reached, the initial Cl content is significant.

Although one cannot accurately compare the permeation of B relative to Cl one can however compare the ratios B/Cl, C/Cl, D/Cl and so obtain a more accurate idea of the relative permeations of B, C and D. Further, if the ratio B/Cl is equal to 1 or is greater than 1, it may be said that B permeates more readily than Cl, while if R is very much less than 1, one may infer that B permeates less readily.

It would, of course, be easy to remove chloride from erythrocytes by a preliminary soaking in potassium sulphate solution and to suspend the chloride-free cells in a mixture of KB and KCl, but it has been found that the permeability of cells twice suspended in salt solutions differs greatly from that of cells only once suspended, an observation which agrees with the experiments of Brinkman and Van Dam [1920].

Method.

Normal human blood was centrifuged in two tubes drawn out into sealed graduated and calibrated capillaries for thirty minutes at 4000 r.p.m. The volume of erythrocytes was noted and the cells in each tube then suspended in one hundred times their volume of the same test-fluid at 23°. This fluid usually contained 87·5 m.eq. of the test-anion (B) and 87·5 m.eq. of chloride. After 5 minutes the tubes were respun, the p_H of the clear fluid determined colorimetrically and the change in volume of the erythrocytes noted. The containing tube was carefully washed free of all trace of salts with distilled water, which was introduced with a capillary pipette down as far as the upper face of the erythrocyte column. The cells were then analysed. Sometimes it was necessary to examine the stability of anion distribution in erythrocytes, and under these circumstances the cells were suspended for periods varying from one to sixty minutes, and the relative anion contents were then compared.

It is clear that errors in reading the p_H of an unbuffered solution are much greater than if the solution is buffered. In fact, however, the external solution is never completely unbuffered, because a trace of bicarbonate diffuses into it from the suspended erythrocytes. To limit p_H errors as far as possible, two solutions of each indicator were used, one at each end of the corresponding p_H range. The method in the case of slightly alkaline KCl solution was as follows. A few drops of slightly acid phenol red were added as a layer on the surface of the clear supernatant fluid in one of the centrifuge-tubes, while to the fluid in the duplicate tube the slightly alkaline phenol red was added. As each indicator diffused downwards, its colour changed slightly, the orange solution becoming slightly redder and the red solution becoming slightly yellow. The weakest clearly perceptible colour in the supernatant fluid was matched with an inverted p_H colour-tube, comparison being made with a layer of suitable thickness in the tapering portion of the standard tube. It was thought that by these means a fair idea of p_H might be obtained.

Chloride was estimated by Claudius's method [1924]; phosphate by the method of Briggs [1922]; iodide and iodoacetate by the method of Kendall [quoted by Harington and Randall, 1929] and lactate by Mendel and Goldscheider's method [1925].

Salicylate was estimated by Benedict and Theis's colorimetric method [1924] slightly modified. Diazotisation was allowed to proceed for fifteen minutes. It was found desirable to prepare numerous standards so that the colour of the unknown did not differ by more than 20 % from that of the standard. Duplicate experiments on diffusion of salicylate into erythrocytes gave results which agreed within 10 %.

Oxalate was estimated by permanganate titration of the protein-free filtrate from cells treated with zinc hydroxide; a blank titration was done on normal cells.

Errors in the method are least marked in buffered solutions and are most evident in neutral unbuffered solutions. This arises from difficulties in reading p_H and from the relatively low chloride content to be estimated.

Errors in unbuffered solutions at p_H 7 are as follows:

Errors in tube volume	$\pm 0{\cdot}5$ %.
Errors in Cl estimation	$\pm 1{\cdot}5$ m.eq. in Y.
Errors in p_H reading	$\pm 0{\cdot}15\ p_H = \pm 7$ m.eq. in X.

Another possible source of error arises from differences in the haemoglobin content of unit volume of cells. In normal blood such differences are slight.

Table IV. *Summary of Results.*

Group	No.	Anion B	p_K		p_H 5·1					p_H 6·0					p_H 7·0				
					% of original vol.	Chloride m.eq.	B m.eq.	B/Cl calculated	B/Cl estimated	% of original vol.	Chloride m.eq.	B m.eq.	B/Cl calculated	B/Cl estimated	% of original vol.	Chloride m.eq.	B m.eq.	B/Cl estimated	B/Cl calculated
Inorganic	1	Chloride	—	—	129	205	—	1·00	—	114	150	—	1·00	—	98	92	—	1·00	—
	2	Phosphate	—	—	126	173	32	0·18	0·05	109	126	24	0·19	0·06	103	80	12	0·15	0·06
	3	Iodide	—	—	116	94	111	1·18	1·20	110	71	79	1·11	1·27	96	41	51	1·24	1·39
	4	Sulphate	—	—	122	126	79	0·63	—	115	112	38	0·34	—	103	76	16	0·21	—
	5	Nitrate	—	—	134	102	103	1·01	—	106	68	82	1·20	—	97	44	48	1·09	—
Organic	6	Thiocyanate*	—	—	134	84	94	1·12	—	103	60	90	1·50	—	93	44	48	1·09	—
ALIPHATIC	7	Formate	—	3·7	136	115	90	0·78	—	112	80	70	0·87	—	100	54	38	0·70	—
Lower fatty acids	8	Acetate	—	4·73	144	79	126	1·60	—	114	80	70	0·87	—	96	50	42	0·84	—
	9	Iodoacetate	—	3·15	136	80	125	1·56	1·75	113	81	69	0·85	1·00	98	52	40	0·77	0·77
	10	Propionate	—	4·8	140	82	123	1·50	—	112	80	70	0·87	—	100	54	38	0·70	—
	11	Butyrate	—	4·8	144	72	133	1·84	—	113	80	70	0·87	—	98	49	43	0·88	—
	12	Valerate	—	4·81	150	68	137	2·02	—	117	74	76	1·03	—	101	54	38	0·70	—
Hydroxy-aliphatic acids	13	Glycollate	—	3·84	130	117	88	0·75	—	110	94	56	0·60	—	97	64	28	0·44	—
	14	α-Hydroxypropionate	—	3·85	130	121	84	0·69	—	112	104	46	0·44	0·33	96	73	19	0·26	—
	15	β-Hydroxypropionate	—	4·61	136	95	110	1·16	—	108	94	56	0·60	—	96	66	26	0·39	—
	16	β-Hydroxybutyrate	—	4·52	137	92	113	1·23	—	113	87	63	0·72	—	101	67	25	0·37	—
	17	Glycerate	—	3·65	120	142	63	0·44	—	106	120	30	0·25	—	90	72	20	0·28	—
	18	Thiolacetate	—	3·55	128	70	135	1·92	—	110	68	82	1·20	—	104	60	32	0·53	—
Ketonic acids	19	Pyruvate	—	2·5	130	106	99	0·94	—	112	92	58	0·63	—	100	66	26	0·39	—
	20	Levulate	—	4·69	141	78	127	1·62	—	114	80	70	0·87	—	98	60	32	0·53	—
Dicarboxylic acids	21	Oxalate	1·42	4·39	105	28	177	6·32	5·60	106	71	79	1·11	1·0	97	48	44	0·92	0·81
	22	Malonate	2·8	5·68	122	110	95	0·86	—	111	94	56	0·60	—	102	68	24	0·35	—
	23	Succinate	4·18	5·57	126	114	91	0·80	—	116	122	28	0·23	—	102	74	18	0·24	—
	24	Azelate*	4·62	5·6	115	72	106	1·47	—	108	74	76	1·03	—	102	75	17	0·23	—
	25	Maleate	1·93	6·58	114	110	95	0·86	—	115	106	44	0·41	—	106	75	17	0·23	—
	26	Fumarate	3·03	4·49	121	138	67	0·49	—	—	—	—	—	—	—	—	—	—	—
	27	Tartrate	3·0	4·39	126	164	41	0·25	—	—	—	—	—	—	103	79	13	0·16	—
Tricarboxylic acid	28	Citrate	—	—	—	—	—	—	—	116	126	24	0·19	—	—	—	—	—	—
AROMATIC	29	Benzoate*	—	4·2	123	72	106	1·48	—	106	60	90	1·50	—	96	49	43	0·88	—
	30	o-Hydroxybenzoate*	—	3·0	112	61	117	1·92	2·07	104	54	96	1·78	1·75	95	44	48	1·09	1·14
	31	m-Hydroxybenzoate	—	4·13	124	70	135	1·93	—	100	54	96	1·78	—	—	—	—	—	—
	32	p-Hydroxybenzoate	—	4·55	122	70	135	1·93	—	102	55	95	1·73	—	—	—	—	—	—
	33	p-Nitrobenzoate*	—	3·47	110	60	118	1·97	—	108	57	93	1·64	—	95	50	42	0·84	—
	34	p-Aminobenzoate	—	—	126	66	139	2·10	—	104	58	92	1·58	—	99	44	48	1·09	—
	35	α-Naphthoate*	—	4·7	110	56	149	2·67	—	102	48	102	2·13	—	93	44	48	1·09	—
	36	Phthalate	2·92	5·41	102	64	141	2·21	—	117	84	66	0·79	—	96	64	28	0·44	—
	37	Cinnamate*	—	4·46	108	59	119	2·02	—	—	—	—	—	—	—	—	—	—	—
	38	Mandelate	—	3·37	128	94	111	1·18	—	106	86	64	0·75	—	94	58	34	0·59	—
	39	Diphenylacetate	—	3·96	—	—	—	—	—	103	59	91	1·54	—	—	—	—	—	—
ALICYCLIC	40	Hexahydrobenzoate	—	4·88	—	—	—	—	—	118	72	78	1·08	—	98	48	44	0·92	—
	41	Quinate	—	3·54	118	156	49	0·31	—	107	123	27	0·22	—	95	75	17	0·23	—

* (At p_H 5·5.)

From a consideration of the values obtained in parallel experiments it is considered that where the ratio of B/Cl is in the neighbourhood of unity, differences exceeding 20 % are significant, but if the ratio is about 0·3, differences of permeation should not be assumed unless the ratios differ by 50 % or more.

The estimated ratio of B/Cl is shown in Table IV immediately after the calculated ratio. The organic anions in the Table are arranged in an aliphatic and an aromatic series, and these are subdivided into the simple anions and those containing additional polar groups, *e.g.* OH, COOH *etc.*

Inorganic anions and thiocyanate.

(Table IV.) The order of permeation is thiocyanate > iodide > nitrate, chloride > sulphate > phosphate.

Phosphate-chloride system. Solutions containing KH_2PO_4 and K_2HPO_4 (175 m.eq.) were mixed to give the required p_H, and an equal volume of KCl was added. It was thought better to use solutions of the same equivalence rather than the same osmotic pressure. Since phosphate may be univalent or bivalent within the p_H range investigated, it is not possible to determine accurately the content of cell-phosphate from the values for cell-phosphorus without knowing the p_H of the cell. But as over 80 % of phosphate is univalent between p_H 5 and 6·2 no serious error is involved if one assumes that phosphate is mainly univalent when the p_H of the external solution is between 5 and 6. In any case the figures for cell-phosphorus are so low as to make it clear that phosphate permeates very much more slowly than Cl (Table V).

Table V.

5 minutes at 23°.

p_H	% of original volume	Cell-Cl m.eq.	Cell-P mg.	P m.eq.	R
4·8	130	147	62	20	0·13
5·1	126	173	26	9	0·05
6·0	109	126	21	7	0·06

It will be noted that phosphate permeates at about the same rate between p_H 5·1 and 6, but at p_H 4·8, where some degree of haemolysis occurs, the phosphorus content rises sharply. It will also be seen that the permeation of phosphate is greatly depressed, while chloride enters to excess to satisfy the combining power of cell-base. Thus cells in pure phosphate solution containing 570 mg. P take up 235 mg. of P in 5 mins. at p_H 5·1, while if the phosphate is diluted with KCl so that its P content becomes 285 mg., cell-phosphorus does not diminish proportionately to 117 mg., for actually the cells take up only 26 mg. of P.

Effect of p_H *on phosphate permeation.* After 5 mins. suspension at 23° cells take up very little phosphate and the content is practically the same at p_H 5 and 6 (24 mg. P), but if the cells are immersed for 1 hour in phosphate-chloride solution at 33° (containing 87·5 m.eq. Cl and 285 mg. P) they take up 85 mg. P at p_H 5·1 and 215 mg. P at p_H 6. In other words if the exposure of erythrocytes to phosphate-chloride mixtures is prolonged, a certain impermeability develops at p_H 5 relative to p_H 6. It is thought that this may be due to a specific coagulation effect of phosphate at low p_H, which greatly decreases the permeation of phosphate itself, while leaving the permeation of the chloride ion relatively little affected.

The unequal distribution of phosphate and chloride inside erythrocytes is unstable, for the longer the cells are exposed to mixtures of phosphate and chloride, the more nearly does the distribution of these anions in the cells become proportional to that in the external solution.

Iodide-chloride (No. 3). Iodide was estimated directly and chloride, *Y*, was calculated indirectly and was also estimated directly from the total halide content of the protein-free filtrate of erythrocytes treated with sodium tungstate and sulphuric acid, silver nitrate and nitric acid being added and the solution titrated with thiocyanate, without previous boiling. The chloride content of the cells was also estimated by Claudius's method, for it was found that after prolonged boiling with $AgNO_3$, $K_2Mn_2O_8$ and HNO_3 the silver iodide was decomposed almost entirely, and a halide value was obtained which equalled the cell-chloride *plus* a small residue of iodide. Thus at p_H 6, iodide was 90 m.eq., giving a calculated Cl value of 60 m.eq. Total halide was estimated as 161 m.eq., giving an actual chloride value of 71 m.eq., which by the second method (acid incineration) was found to be 74 m.eq. At p_H 7, chloride was calculated at 35 m.eq. compared with estimated values of 41 and 39 m.eq.

It is clear that iodide attains a greater concentration in erythrocytes than does chloride, and this is the more remarkable when it is recalled that before iodide diffusion has begun chloride is already at its natural level of 50 m.eq. Moreover the unequal distribution is relatively stable for periods up to one hour; at p_H 5·9 cell-chloride and iodide were respectively 67 and 93 m.eq. while after 1 hour they were 69 and 96 m.eq.

Sulphate-chloride (No. 4), sulphate permeates less readily than chloride; this is least evident at p_H 5·1 and very marked at p_H 7.

Organic anions.

A large number of organic anions have been examined. Most of them have been standardised by titration, but in some cases salts of known purity were used. In the following, calculated (as opposed to estimated) values of B are shown in brackets.

All the organic anions appear to penetrate more readily at low p_H.

Lower fatty acids. This group, from acetate to valerate, appears to permeate more readily than chloride at p_H 5·1 and at about the same rate or very slightly more slowly between p_H 6 and 7. Table IV shows some indication that permeation becomes more rapid with increasing length of the carbon chain.

Formate (No. 7) is exceptional, for while between p_H 6 and 7 it has the same ratio as the acetate-valerate series, it permeates much more slowly at p_H 5·1.

Acetate-chloride (No. 8) (sodium acetate; KCl and HCl). At p_H 5·1, *R* is 1·60; at p_H 4·9, however, some haemolysis occurs, and *R* falls to 0·83. Presumably the damage to the cell membrane impairs its semipermeability so that the superior penetration of acetate is no longer in evidence.

Differences in permeation between acetate and chloride at p_H 5 and 6 are clearer if acetate and chloride are mixed in the proportion of 1 : 7 and cells are suspended for 5 and 60 mins. At p_H 6, at which acetate and chloride permeate at about the same rate, the distribution of acetate and chloride remains unaltered. At p_H 5·1, the superior permeation of acetate is shown by the low initial chloride at 5 mins. and its rapid increase during the subsequent 55 mins. (Table VI).

In order to confirm the superior permeation of acetate, an iodoacetate-chloride system was used, although the insertion of an iodine atom in the CH_3 group may well alter the permeation of the organic anion. However, the comparative similarity of the observed iodoacetate value and the calculated acetate

Table VI.

	External acetate 22 m.eq. p_H 5·1		External chloride 153 m.eq. p_H 6	
	Cell-chloride	Cell-acetate	Cell-chloride	Cell-acetate
5 mins.	107	(98)	129	(21)
60 mins.	170	(35)	131	(19)

figure lends support to the belief that the latter is approximately correct (Table IV, Nos. 8, 9).

Hydroxyaliphatic acids. The following were examined: glycollate, lactate, β-hydroxypropionate and β-hydroxybutyrate; these substances also permeate most rapidly at p_H 5·1 and more slowly at higher p_H. The addition of a hydroxyl group decreases penetration, and this decrease is much greater when the OH is in the α-position than when it is in the β-position. Indeed at p_H 5·1 the permeation of the β-hydroxy-anion is greater than that of Cl, but at p_H 7 it is probably less.

Lactate. The calculated values for lactate were checked by the veratrol colorimetric method. At p_H 5·05, lactate was calculated to be 89 m.eq. and estimated as 84 m.eq.; at p_H 5·7, lactate calculated was 44 m.eq. and estimated to be 38 m.eq.

The superior permeation of β-hydroxy- over α-hydroxy-anions is shown by a comparison of the B/Cl ratio at 5 and 60 mins. In the lactate mixture, the chloride which permeates first is displaced by the organic anion and decreases with time. In the β-hydroxypropionate mixture, chloride increases towards an equable distribution of anions.

Table VII.

(Ext. Cl 87·5 m.eq., Ext. B 87·5 m.eq.)

	Lactate p_H 5·1			β-Hydroxypropionate p_H 5·05		
	Cl	B	*R*	Cl	B	*R*
5 mins.	118	(87)	0·74	96	(114)	1·19
60 mins.	112	(93)	0·83	107	(103)	0·96

Glycerate (No. 17). This dihydroxy-acid permeates much more slowly than the preceding anion.

Thiolacetate (No. 18). The substitution of sulphur for the oxygen in the polar group increases permeation.

Ketonic acids. These acids like the hydroxy-acids also permeate less quickly than the corresponding fatty acids, and similarly when the carbonyl group is near the —COOH group permeation is slower than when it is further removed from the carboxyl group. Compare pyruvate with laevulate, which has the CO group in the γ-position (Nos. 19 and 20).

Amino-acids. The dissociation constants of these substances render them unsuitable for examination.

The dicarboxylates. These fall into two groups; oxalate which permeates more quickly than Cl, and the other dicarboxylates which on the whole permeate more slowly. The latter include the saturated malonate and succinate and the unsaturated maleate and fumarate. It also includes the hydroxy-anion tartrate.

All these acids have two dissociation constants, and they were adjusted to give a constant concentration of 87·5 m.eq. when mixed with an equal volume of KCl solution. Table IV shows that all these acids permeate more readily at low p_H; that they permeate less readily than oxalate; that succinate permeates more slowly than malonate, and that they all permeate more slowly than Cl at p_H 7;

that fumarate which is mainly bivalent at p_H 5·1 permeates less readily than maleate which is mainly univalent at this p_H. The table shows that the addition of hydroxyl or carboxyl groups to dicarboxylates makes it almost impossible for the anion to penetrate the cell membrane (*e.g.* tartrate (No. 27) and citrate (No. 28)). It must be noted however that azelate (No. 24), $(CH_2)_7(COOH)_2$ penetrates rapidly between p_H 5·5 and 6. It is thought that this may be correlated with its high solubility in organic solvents and its low dissociation.

Oxalate-chloride (No. 21). Oxalate was considered as a dibasic acid (p_K 1·42 and 4·39) at p_H 5·1. p_H was adjusted with oxalic or hydrochloric acid and in either case similar results were obtained.

Oxalate is unique in that it permeates more rapidly than chloride or any other anion at low p_H, and at p_H 7 it permeates more quickly than most anions and probably as quickly as chloride, for at p_H 7 cell-chloride remains practically unaltered while oxalate mounts from 0 to 39 m.eq.

The effect of length of exposure on the chloride-oxalate distribution at p_H 5·1 and 6·1 is as follows:

Table VIII.

	p_H 5·1			p_H 6·1		
Time	Cell-Cl	Cell-oxalate	R	Cell-Cl	Cell-oxalate	R
0 min.	50	0	0	50	0	0
¼ min.	23	131	5·7	38	104	2·7
1 min.	26	139	5·4	—	—	—
5 mins.	30	168	5·6	69	70	1·0
60 mins.	83	126	1·5	—	—	—

So rapidly does oxalate diffuse that in a few seconds its concentration is six times as great as that of chloride. With the passage of time however cell-Cl rises and oxalate falls so that equality is approached but is not nearly attained in one hour. At p_H 6 equality of distribution is reached in 5 mins., but even here the superior permeation of oxalate can be shown by exposing cells to oxalate-chloride solution for only a few seconds, when it will be found that oxalate has increased to 104 m.eq. while chloride has decreased to 38 m.eq.

While superior permeation of oxalate explains its high initial cell concentration followed by its subsequent decrease, it does not explain the corresponding changes in cell-chloride, which falls from 50 to 23 m.eq. in a few seconds and then rises at 1 hour to 83 m.eq. This can only be due to an actual displacement of chloride from the cell followed by its return into the cell again.

For this power of displacing chloride there seems to be no adequate explanation.

The oxalate-chloride system also illustrates the depression of chloride permeation by the more mobile anion. Cells in pure KCl at 175 m.eq. at p_H 5·1 take up 205 m.eq. Cl. In a mixture containing 87·5 m.eq. each of oxalate and chloride, the cells instead of taking up 102 m.eq. Cl actually lose 30 m.eq. and give a Cl content of about 20 m.eq. Even if cells are placed in a mixture containing 155 m.eq. Cl and only 22 m.eq. of oxalate at p_H 5·1, they take up only 58 m.eq. of chloride and as much as 115 m.eq. of oxalate in 5 mins.

A similar unequal distribution of oxalate and chloride (and also of acetate and chloride in the case of acetate-chloride mixtures) is obtained if freshly collected unheparinised blood is added directly to the salt mixtures.

It should be noted with regard to oxalate that it must be numbered among those anions which decrease permeation and cause shrinkage of cell volume at

low p_H. Thus in oxalate-chloride mixtures, cell volume at p_H 5·5 is 111 %; at p_H 5·1, 105 % and at p_H 4·5, 102 %, while the corresponding total anion contents are respectively 168, 198 and 167 m.eq.

Aromatic anions.

Benzoate permeates rather more readily than chloride, but the following anions, *o*-, *m*- and *p*-hydroxybenzoate, nitrobenzoate and *p*-aminobenzoate, though containing a carboxyl group and an additional polar group, all permeate more readily than benzoate itself. This superior permeation is most marked at low p_H.

Phthalate (No. 36), which contains two carboxyl groups, permeates rapidly at p_H 5·1 but at p_H 7 its entry is slower than that of any of the preceding anions and probably slower than that of chloride itself.

Naphthoate (No. 35), which contains two benzene rings, penetrates with great speed and can be qualitatively recovered from the erythrocyte. Experiments with naphthoate, nitrobenzoate and cinnamate were carried out at p_H 5·5, 6 and 7, and acidity was adjusted by the addition of phosphate solution to equal 25 m.eq. in the final mixture. In this way precipitation of the acid at p_H 5·5 was avoided while, owing to its slow permeation, effects due to phosphate itself could be neglected.

Salicylate-chloride. Salicylate was determined directly and indirectly. The salicylate-chloride values are shown in Table IV (No. 30). The unequal distribution of these anions is not entirely stable, but even after erythrocytes have been suspended in salt mixtures for 30 mins. at 33° and after haemolysis has occurred, it is very evident that salicylate is still present in a concentration which is much higher relatively than Cl (Table IX). This is most clearly seen when the external solution contains 153 m.eq. of chloride and only 22 m.eq. of salicylate (Table IX).

Table IX.

p_H 5·7.

Time	Temp.	External Cl	External salicylate	Cell-Cl	Cell-salicylate
¼ min.	23°	153	22	56	91
5 mins.	23°	153	22	63	93
30 mins.	23°	153	22	82	68
60 mins.	33°	153	22	120	50

Three of the anions examined contained benzene rings attached to an aliphatic side-chain; of these cinnamate and diphenylacetate permeated quickly. But mandelate (No. 38) which contains a hydroxyl group in the aliphatic chain resembles the aliphatic anion glycollate in its penetrating power and not salicylate (No. 30) which contains the hydroxyl group attached to its benzene ring.

The permeation of the saturated alicyclic anions, hexahydrobenzoate and quinate, resembles that of the aliphatic acids, in that the former permeate quickly, while the latter hardly penetrates at all.

Discussion.

The view is generally held that the erythrocyte consists of a semipermeable envelope holding in its interior water, haemoglobin, salts and possibly lipin. The envelope contains lecithin, cholesterol, probably haemoglobin and also a stroma protein, isoelectric at p_H 5·5, described by Jorpes [1932].

Erythrocytes are impermeable to cations at $p_H < 8$, and this indicates that the cell membrane must be positively charged [Mond, 1928]. On the other hand, cataphoresis shows that the surface is negatively charged [Höber, 1904]. But as Mond remarks, the cell membrane may consist of two elements; an electronegative, giving the cell its cataphoretic properties, and a positively charged portion making the cell impermeable for cations.

The present investigation is concerned with the passage of anions through this complex envelope. The work of Henderson [1928] and of Van Slyke on electrolyte equilibria and the experiments of Höber [1910] on conductivity show that within the erythrocyte chloride is mainly ionised, and as it has been found that other anions quantitatively replace chloride, it may be presumed that these are also ionised. Further it has been found that erythrocytes take up anions from acid salt solutions, and as the process is accompanied by cell swelling, it seems probable that the bulk of cell-anion is distributed within the erythrocyte and not on its surface.

The difference of acid distribution within erythrocytes may depend on differing degrees of unionised complex formation or on differing ionic activities. In this case, one would expect the inequality of distribution to be stable. On the other hand unequal distribution may be due to differing rates of permeation through the cell membrane, in which event the inequality would be maximum at first and tend to diminish subsequently.

The only systems showing a stable inequality are iodide-chloride and thiocyanate-chloride, and the work of Hastings and Van Dyke [1931] on bromide-chloride distribution suggests that this may be due to iodide forming unionised compounds to a greater extent than chloride. In the case of salicylate-chloride, where the initial disparity in distribution diminishes to a fairly stable inequality, it is thought that both of the above mechanisms may be concerned. Most of the other systems show an unstable inequality which tends towards ultimate equality of anion distribution within the cell, and this is ascribed to differences of permeation through the cell membrane.

In view of the large size of some rapidly penetrating ions and the small size of others that permeate slowly, it seems unlikely that the pore theory of membrane permeability can hold with regard to the erythrocyte envelope, a probable alternative being that permeation rate depends on relative solubility in the cell membrane. It seems clear from Table IV that permeation does not depend on the solubility of anions in water, and the theory of Overton seems more applicable, namely that the penetration of substances into cells depends partly on their solubility in oily or lipoid substances. According to Jacobs [1924] the substances investigated by Overton fall into two groups—polar and non-polar. The latter (*e.g.* simple hydrocarbons) are unionised and soluble in organic solvents; these are said to penetrate cells rapidly; the former which contain groups like —OH, $—NH_2$ or —COOH tend to be dissociated and water-soluble; these penetrate cell membranes less readily, and the more numerous the polar groups, the more slowly does permeation occur. Attempts to support or refute these theories are necessarily inconclusive in the absence of data regarding the solubility of substances in the cell membrane. However, the permeation of substances into erythrocytes has been examined from the standpoints of their dissociation and their partition coefficients in an ether-water system.

Reference to Table IV shows that the simple aliphatic acids with one polar —COOH group permeate more rapidly than those with two polar groups, whether there be an —OH or an additional —COOH group, while substances like glycerate, tartrate and citrate (Nos. 17, 27 and 28) which have more than two

polar groups penetrate still more slowly. All these substances with numerous polar groups are more strongly dissociated and concentrate to greater extent than the corresponding fatty acids in the water phase of an ether-water system [Smith, 1921].

Overton suggests that organic substances permeate in the undissociated state, and it seems probable that the permeation of organic acids in general may depend on the dissociation constants of the acid and on its oil-solubility, varying according to the resultant of these two factors. Thus in the simple fatty acid group, the partition coefficient of formic acid is less than that of acetic acid, and the former is much more strongly dissociated, and it penetrates much more slowly at p_H 5·1, resembling the hydroxy-aliphatic acids in this. But at p_H 7, where both acids are well dissociated, the penetrations of formate and acetate are similar.

The members of the acetate-valerate series dissociate to an equal extent, but there is a marked increase in ether- and olive oil-solubility as the length of the carbon chain increases. The apparent increase of permeation however is only slight, but probably changes in the B/Cl ratio are masked by pre-existing chloride in erythrocytes.

The α-hydroxy-acids, glycollate and lactate are strongly dissociated and concentrate in the water phase; these substances penetrate much more slowly; the β-hydroxy-acids on the other hand are weak acids and, although their partitions are similar to those of the α-acids, their penetration is more rapid. The same applies to pyruvic and laevulic acids. In the former, the ketonic group is next the carboxyl; it is well dissociated and penetrates slowly; in the latter a γ-ketonic group is associated with low dissociation and more rapid penetration, although the partition coefficients of pyruvic and laevulic acids are similar.

Both the hydroxy- and keto-acids permeate more slowly than the corresponding fatty acids at higher p_H.

The dicarboxylic acids, malonic and succinic, are moderately well ionised at p_H 5·1, they concentrate in the water phase and their permeation is slow. But azelaic acid, $(CH_2)_7(COOH)_2$, on the other hand is less well dissociated and has an ether-water partition of 9·5. At p_H 5·1 it permeates rapidly, but at p_H 7, its permeation is slow. In this series, oxalate is exceptional, and it is not possible to correlate this with its power to precipitate calcium, for there is no detectable calcium in the erythrocyte, while even if calcium precipitation resulted in phase reversal of the cell membrane, there is no reason why oxalate should permeate more rapidly than the water-soluble anion, chloride or the ether-soluble organic acids.

In the case of the aromatic series, even complex substances like naphthoic and diphenylacetic acids permeate rapidly, and the presence of two polar groups as in the hydroxy-, amino- and nitro-benzoic acids does not seem to interfere with permeation. All these substances concentrate in the ether phase and attain a relatively high concentration in the oil of an olive oil-water system, and all quickly penetrate the erythrocyte membrane. It may be noted that while the presence of an —OH group in the benzene ring does not delay permeation, the substitution of —H by —OH in the aliphatic portion of phenylacetate greatly depresses permeation, which becomes comparable with that of the hydroxy-aliphatic acids: thus the permeation of mandelate is similar to that of lactate. It may be observed that the p_K, ether-water partition and permeation ratio at p_H 6 of salicylic acid are respectively 3·0, 5·0 and 1·78, while the corresponding data for mandelic acid are 3·37, 1·33 and 0·75.

The effect of p_H *on permeation.* The permeation of anions relatively to chloride decreases as p_H increases. This is due partly to the chloride which is normally present in erythrocytes, and the effect will be more apparent at high p_H, where the total anion taken up by the cells is less. But that the ratios obtaining at p_H 5·1 are not only due to this effect may be seen if the chloride figures at p_H 5·1 and 6 are compared. For not only does B increase as the total cell-anion increases, but in many cases cell-chloride remains unaltered and may even decrease, while in all cases the increase of B between p_H 6 and 5 is greater than the corresponding increase in chloride. This relative increase in permeation of anion B may be due to its being less dissociated at low p_H, but such an explanation could not apply to sulphate whose permeation relatively to chloride is also higher at p_H 5·1 than at 6. It might be thought that swelling of the cells would itself increase permeation by stretching the cell membrane, increase of permeation occurring *pari passu* with swelling. That this is not the case may be shown by suspending erythrocytes in hypertonic and hypotonic lactate-chloride solutions at p_H 7; in the latter solution, marked cell swelling occurs but the ratio of cell-lactate to chloride remains the same.

If one conceives the erythrocyte membrane as a negatively charged protein sieve whose holes are filled with lipoid material, then it follows that the restraining effect of the sieve protein meshwork on the permeation of anions will be decreased at low p_H. Further at low p_H the cell swells but the protein element will contract as it approaches its isoelectric point and as the total area of the cell membrane increases, it follows that the lipoid element must increase in area and decrease in thickness; this will differ from the effect of swelling resulting from hypotonicity where the protein and lipoid elements will be extended equally.

It is also possible that p_H may affect permeation directly through the lipoid portion. Condensed monomolecular films become expanded at low p_H [Adam, cited by Rideal, 1930] and such a change in the erythrocyte membrane may well alter its permeability. In this connection reference may be made to the high temperature coefficient of expansion of unimolecular fat films—a finding which is comparable in degree and range with the temperature coefficient of phosphate diffusion into erythrocytes [Maizels, 1932].

It is not suggested that lipoid-solubility is the only factor in, or even the direct means of, cell permeation, for adsorption, as well as solubility in organic solvents, is an expression of hydrophobe qualities. It is also possible that adsorption and permeation may occur at the protein-water interface of the cell envelope. Indeed it is likely that water-soluble substances and water itself may penetrate by this route.

Summary.

1. The order of permeation of inorganic ions into erythrocytes is (thiocyanate) > iodide > nitrate, chloride > sulphate > phosphate—a lyotropic series.

2. The permeation rate of anions relative to chloride increases with the external hydrogen ion concentration.

3. The presence of additional polar groups decreases the permeation of organic anions. This effect is less marked, if the polar group is not in the α-position to the carboxyl group, and it also becomes less marked with increasing length of the carbon chain.

4. Substances permeating rapidly are usually poorly dissociated, have a relatively high ether-water partition coefficient and are capillary-active.

REFERENCES.

Benedict and Theis (1924). *J. Biol. Chem.* **61**, 67.
Briggs (1922). *J. Biol. Chem.* **53**, 13.
Brinkman and Van Dam (1920). *Biochem. Z.* **108**, 35.
Claudius (1924). *Acta Med. Scand.* **61**, 4.
Harington and Randall (1929). *Quart. J. Pharm. Pharmacol.* **2**, 501.
Hastings and Van Dyke (1931). *J. Biol. Chem.* **92**, 13.
Henderson (1928). Blood. (A study in general physiology.) (Yale University Press.)
Höber (1904). *Pflüger's Arch.* **101**, 607; **102**, 196.
—— (1910). *Pflüger's Arch.* **133**, 237.
Jacobs (1924). General cytology, Section 3. (Chicago.)
Jorpes (1932). *Biochem. J.* **26**, 1438.
Maizels (1932). *J. Physiol.* **77**, 22 P.
Mendel and Goldscheider (1925). *Biochem. Z.* **164**, 163.
Mond (1928). *Pflüger's Arch.* **220**, 69.
Rideal (1930). Surface chemistry. (Cambridge University Press.)
Smith (1921). *J. Physical Chem.* **25**, 605.

XLVI. DILATOMETRIC STUDIES IN THE PROTEOCLASTIC DEGRADATION OF PROTEINS.

I. TRYPTIC HYDROLYSIS.

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The enzymic digestion of complex substances like proteins is attended by a series of physical and chemical changes each of which proceeds at varying rates at different stages of the reaction. The viscosity change of the protein-protease system, for example, proceeds rapidly in the early stages of the reaction, while the changes in optical rotation or in the concentration of amino-groups during the same period are not correspondingly large. Each of these estimations is thus predominantly a measure of the particular type of change occurring during a definite phase of the reaction.

For obtaining an intimate knowledge of the mechanism of protein hydrolysis, it is necessary not only to utilise all the methods that are now available but also to invoke the aid of new technique which would either reveal changes not recorded by other means or independently confirm those indicated by other methods. The dilatometer, therefore, offers a new method of attack in the investigation of the hydrolytic cleavage of complex bodies in general and proteins in particular.

From the colloid chemical point of view, the degradation of the protein molecule can be looked upon as a process in which the degree of aggregation of the substrate is being diminished, the few colloidal micellae giving rise to several crystalloidal molecules varying in magnitude and composition. This process is accompanied by a disturbance in the water equilibrium of the system. From the chemical point of view the hydrolysis can be considered as a reaction involving the progressive release of amino- and carboxyl-groups.

Each of the above changes brings about a positive or negative volume change, the combined result of which is registered by the dilatometer. In order to resolve and apportion the net dilatometric change to its constituent physical and chemical reactions, the volume change specific to each of them has to be determined. These relationships can be ascertained by following the reaction during that phase of hydrolysis at which the change in question proceeds either predominantly or exclusively. When such a choice of phase is not possible, the object can be achieved by adopting such experimental conditions that one definite reaction proceeds to the practical exclusion of others. One of the possible ways of accomplishing this is to fractionate the enzyme complex into simpler constituents and investigate their action on the protein.

Galeotti [1911] has conducted a dilatometric study of the tryptic hydrolysis of peptone and milk. The reacting fluids are subject to changes in surface tension which would affect the change in the capillary column of the dilatometer. The tryptic digestion of milk would really constitute at least two definite systems, *viz.* milk-fat-lipase and caseinogen-trypsin, so that the observed volume change would not measure any one specific reaction.

While the present investigation was in progress, Rona and Fischgold [1933] have published a paper on the peptic digestion of ovalbumin and caseinogen in which they have tried to correlate the NH_2 release with volume change. A linear relationship was found to exist between the release of amino-groups and the dilatometric depression in the case of ovalbumin; such a relationship, however, was not obtained in the case of caseinogen. The experimental procedure adopted by Rona and his collaborators does not lend itself to a study of the interesting changes which occur during the first thirty minutes of the reaction. With the new type of dilatometer described in one of our earlier communications [1932] it is possible to investigate these changes accompanying an enzymic hydrolysis from its very commencement.

The present communication deals with the dilatometric study of the tryptic hydrolysis of caseinogen and gelatin. The reaction has been followed by an entirely independent method, *viz.* determination of amino-nitrogen by Van Slyke's method.

Materials. Hammerstein's "casein," Gold label gelatin and Pfansteil's trypsin (1 : 75) were the materials employed in the present investigation. All the reactions were conducted at p_H 7·7, employing Sørensen's phosphate buffer, and at a temperature of 30°. Caseinogen and gelatin digestions have been carried out at two different concentrations, and the experimental procedure adopted is similar to that fully described in our previous communication [1932].

Discussion.

The dilatometric fall is expressed in mm.^{3} and the amino-nitrogen in mg. of nitrogen, and the data for each of the systems are graphically represented in Figs. 1 and 2, showing the course of hydrolysis of gelatin and caseinogen as followed by the dilatometer (Fig. 1) and the determinations of the amino-nitrogen (Fig. 2).

The general shape of the two corresponding sets of time-course curves, indicates that the kinetics of tryptic digestion can be followed in the dilatometer as well as by the determinations of amino-nitrogen. The dilatometric method offers a more elegant and less cumbersome technique for studying these reactions; by virtue of the ease and rapidity with which measurements can be conducted, the method possesses the additional advantage that, in the early stages, when enzymic reactions proceed rapidly, a greater number of readings could be obtained.

For the two systems investigated the relation between dilatometric depression and the corresponding increase in amino-nitrogen at different stages of the reaction (calculated from the graphs) is given in Fig. 3. The dilatometric fall in mm.^{3} per millimol release of amino-nitrogen at different time intervals is given in Table I.

A close study of the Table and graphs will reveal the existence of an interesting series of relationships between dilatometric change and release of amino-groups. During the first thirty minutes there is no proportionality between volume change and amino-nitrogen, the dilatometer registering in

a given time a greater or smaller amount of change in proportion to the amino-nitrogen released during the same interval. It will be observed from Table I that the dilatometric depression per millimol release of amino-nitrogen in the case of caseinogen is greater in the initial stages, decreases with

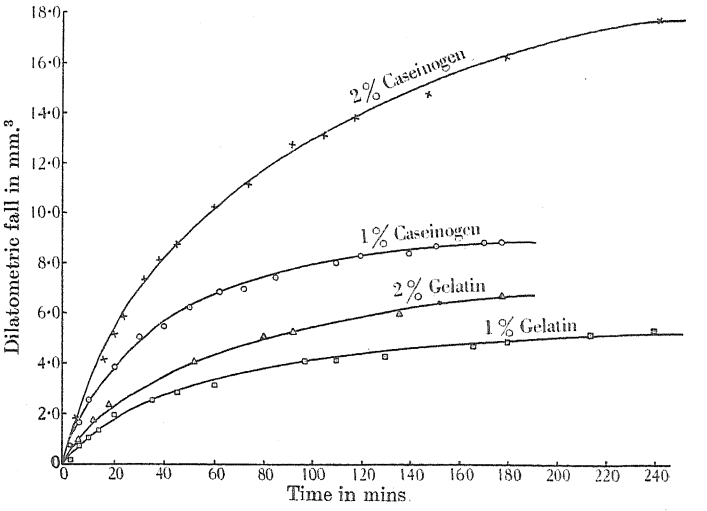

Fig. 1.

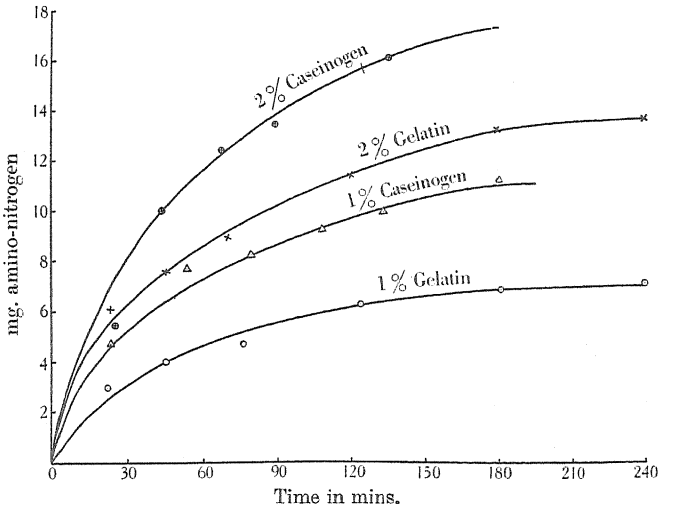

Fig. 2.

the progress of hydrolysis and attains practically a steady value after about 30–40 minutes. In the case of gelatin, however, the ratio increases to a steady value.

This fact is clearly borne out by the graphs (Fig. 3) where an attempt has been made to correlate volume changes with the formation of amino-nitrogen. It will be seen that a strictly linear relationship exists between them after about

Table I.

Time (mins.) ...	10	20	30	40	60	90	120
Dilatometric depression (mm.³) per millimol amino-N.							
Gelatin 1 %	8·8	9·3	9·7	9·8	9·8	9·7	9·7
Gelatin 2 %	6·1	6·5	6·6	6·9	7·1	7·0	7·1
Caseinogen 1 %	13·4	12·6	12·4	12·4	12·3	12·4	11·6
Caseinogen 2 %	16·0	14·8	14·0	13·5	12·8	12·4	12·4

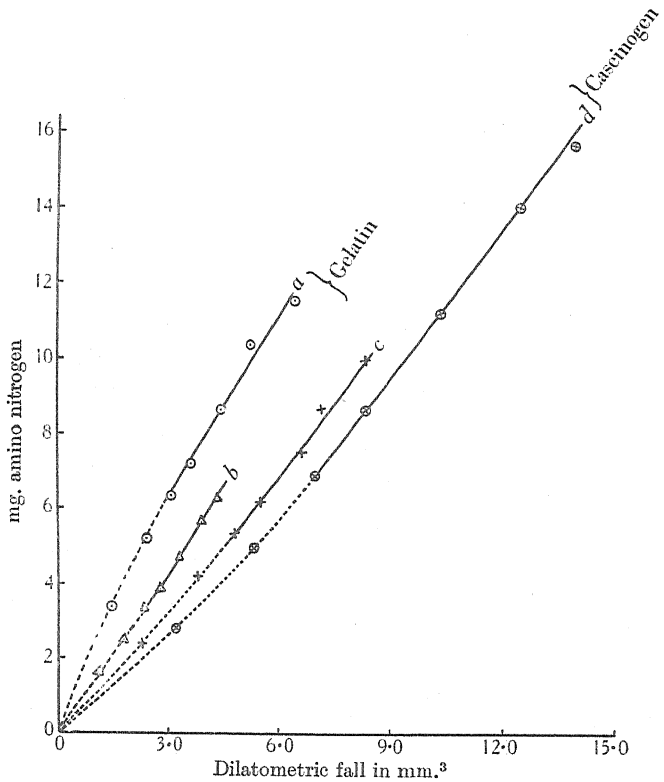

Fig. 3. *a*. 2 % gelatin. *b*. 1 % gelatin. *c*. 1 % caseinogen. *d*. 2 % caseinogen.

30 minutes in the cases both of gelatin and caseinogen. The magnitude of the initial change exclusively recorded by the dilatometer appears to be influenced by the relative concentrations of the enzyme and substrate and is closely connected with the process of liquefaction which generally precedes the rapid cleavage of proteins. A detailed study of this phenomenon is being made.

If the abnormal change occurring in the first stage of the reaction is ignored, a strict proportionality can be observed to exist between the dilatometric depression and the release of amino-nitrogen as shown by Fig. 3. The dilatometric depression per millimol release of amino-nitrogen, which is a constant for each protein, appears to be intimately associated with the structure and amino-acid make up of the protein. Gelatin has a constant of 8·7 while that of caseinogen is 10·8.

SUMMARY.

1. The course of the tryptic digestion of caseinogen and gelatin has been followed in the two-bulbed dilatometer which enables the reaction to be investigated from its very commencement.

2. The early stages of the reaction, during the first 30 or 40 minutes, are accompanied by changes registered by the dilatometer but not indicated by the determinations of amino-nitrogen. After this period, however, the dilatometric depression is proportional to the release of amino-nitrogen in the case of both the proteins.

3. The abnormality occurring in the initial stages of the digestion is closely connected with the process of liquefaction which generally precedes the rapid cleavage of proteins.

4. The dilatometric depression per millimol release of amino-nitrogen has been calculated and found to be 8·7 mm.^{3} in the case of gelatin and 10·8 in the case of caseinogen. This constant appears to be a function of the structure and amino-acid make up of the protein.

REFERENCES.

Galeotti (1911). *Z. physikal. Chem.* **76**, 105.
Rona and Fischgold (1933). *Biochem. Z.* **261**, 366.
Sreenivasaya and Sreerangachar (1932). *J. Indian Inst. Sci.* **15** A, 17

XLVII. THE INFLUENCE OF THE GONADS ON PROTEIN METABOLISM.

IV. THE EFFECT OF OVARIECTOMY AND OF INJECTIONS OF GONADAL AND ANTERIOR PITUITARY EXTRACTS ON URINARY CREATININE IN FEMALE RABBITS.

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The experiments on males detailed in the first three papers of this series [Schrire and Zwarenstein, 1932, 1, 2; 1933] were repeated on females. Creatine and creatinine were determined by Folin's method. The daily output of creatinine remained constant within comparatively narrow limits. The excretion of creatine, on the other hand, varied considerably. In all Tables, Rabbits Nos. 1, 2 and 3 are the normal animals and Nos. 4, 5, 6 and 7 the ovariectomised animals.

Ovariectomy.

In Table I the dates refer to the first of five 24-hour periods in each case. The mean and standard deviation are of the daily creatinine output in mg. given for each series of 24-hour periods. The weights of the animals at the beginning and at the end of the experiments are included.

Table I. *Daily urinary creatinine, mg.*

	Normal			Ovariectomised			
	1	2	3	4	5	6	7
Weight, kg.	2·08	1·96	2·0	2·22	1·96	1·89	1·98
Date			A. Pre-ovariectomy periods.				
25. i. 32	65 ±4·6	—	76 ±1·9	72 ± 5·6	77 ±2·6	74 ±1·4	66 ±4·0
8. ii. 32	77 ±7·4	71 ±4·1	77 ±4·0	76 ± 3·6	74 ±6·0	72 ±4·2	79 ±2·1
15. ii. 32	70 ±3·6	67 ±1·9	74 ±3·9	76 ± 3·6	68 ±5·5	73 ±6·6	75 ±2·8
22. ii. 32	75 ±3·4	70 ±3·3	77 ±3·8	75 ± 7·2	68 ±5·3	74 ±4·0	75 ±1·0
			Nos. 4, 5, 6, 7 ovariectomised 9. iii. 32.				
			B. Post-ovariectomy periods.				
21. iii. 32	—	—	70 ±4·2	79 ± 3·6	—	67 ±3·9	76 ±3·2
18. iv. 32	—	—	71 ±3·9	82 ± 3·0	—	68 ±1·0	74 ±6·4
9. v. 32	—	—	57 ±4·6	74 ± 2·9	—	66 ±4·1	64 ±6·8
6. vi. 32	—	—	56 ±4·8	76 ± 5·5	—	62 ±4·5	67 ±2·8
4. vii. 32	—	—	74 ±6·3	76 ±10·5	—	68 ±3·7	75 ±8·7
13. viii. 32	—	78 ±5·6	78 ±5·1	67 ± 3·1	68 ±3·9	66 ±3·7	68 ±3·4
5. xii. 32	—	90 ±2·5	86 ±4·5	108 ± 8·5	99 ±8·2	98 ±3·5	89 ±6·3
19. xii. 32	92 ±3·7	98 ±7·0	88 ±4·8	104 ± 6·2	99 ±4·5	105 ±5·1	99 ±3·5
9. i. 33	84 ±2·2	92 ±2·7	87 ±3·6	102 ± 2·5	104 ±4·9	99 ±6·5	103 ±6·3
Weight, kg.	3·13	3·01	2·32	3·06	2·72	2·50	2·44

The data in Table I are summarised in Table II in the form of percentage increases in creatinine and body weight.

Table II.

	1	2	3	4	5	6	7
Creatinine	22	31	22	40	44	46	35
Weight	50	54	16	38	39	32	33

Inspection of the data shows that five months after ovariectomy the creatinine excretion was still normal. This result is in striking contrast to the effect of castration in males in which a post-castration rise in creatinine begins three to four months after removal of the testes.

Nine months after the date of ovariectomy an increased creatinine excretion occurred in all animals but the rise was significantly larger in the ovariectomised animals. The increases in creatinine output bore no constant relation to the increases in body weights. A comparison of the percentage figures for the normal and ovariectomised animals shows that the increase in creatinine excretion and gain in weight bore an inverse relation to each other for the two series of animals. The post-ovariectomy rise in creatinine was still maintained 12–14 months after the operation (Tables III and IV).

Table III. *Daily urinary creatinine, mg.*

	Normal			Ovariectomised			
Date	1	2	3	4	5	6	7
13. iii. 33	89	87	87	96	105	100	105
14. iii. 33	92	90	91	116	110	96	105
15. iii. 33	87	91	87	108	98	100	104
16. iii. 33	96	90	88	110	105	105	110
17. iii. 33	100	105	—	100	105	110	109
18. iii. 33	88	80	88	84	97	100	85
19. iii. 33	91	88	90	95	100	95	88
20. iii. 33	96	100	91	105	100	91	110
21. iii. 33	83	80	95	112	95	90	100
22. iii. 33	80	95	85	100	85	80	84
23. iii. 33	84	91	91	100	95	100	110
24. iii. 33	90	88	85	110	105	100	112
17. iii. 33	No injection	Ovary extract 10 cc.	No injection	Ovary suspension	Brain suspension	Brain extract 10 cc.	Ovary extract 10 cc.
21. iii. 33	Testis extract 20 cc.	Testis extract 20 cc.	No injection	Brain extract 10 cc.	Ovary extract 10 cc.	Testis extract 20 cc.	Testis extract 20 cc.

Table IV. *Daily urinary creatinine, mg.*

	Normal			Ovariectomised			
Date	1	2	3	4	5	6	7
24. iv. 33	95	90	88	105	116	110	105
25. iv. 33	100	95	81	116	110	110	116
26. iv. 33	88	91	90	116	105	100	116
27. iv. 33	89	88	90	105*	105*	99*	116*
28. iv. 33	91	100	89	95	89	70	110
29. iv. 33	95	95	91	89	95	105	110
30. iv. 33	91	88	85	100	96	100	110
1. v. 33	95	91	84	100	105	105	110

* 27. iv. 33 Nos. 4, 5 and 6 injected with saline suspension of ovaries.
No. 7 injected with saline suspension of brain.

Injection of saline suspensions of ovaries and of ovarian and testicular extracts.

Finely divided ovaries of cats or sheep and pigs were suspended in Locke's solution and injected subcutaneously. Ovarian and testicular extracts were prepared from sheep's or pig's ovaries or testes by the method of Moore, McGee and co-workers [McGee *et al.*, 1928; Moore *et al.*, 1930]. The final extract was taken up in olive oil before injection. Suspensions or extracts of brain were used as controls. 1 ml. of extract represents 93 g. testis, 58 g. ovary and 91 g. brain.

Injection of saline suspension of ovary into ovariectomised animals caused a decreased excretion of creatinine. In animal 6, Table IV, there was a fall to the pre-ovariectomy level. Both ovarian and testicular extracts caused a slight but probably not significant fall when injected into normal animals. Larger decreases occurred on injection into ovariectomised animals. In all cases brain suspension or extract had no effect.

Injection of anterior lobe extracts.

An extract of anterior lobe of sheep pituitary was prepared according to Bellerby's method [1933]. An extract of brain prepared by the same method served as a control. Both extracts were injected subcutaneously.

Table V. *Daily urinary creatinine.*

	Normal			Ovariectomised			
Date	1	2	3	4	5	6	7
10. v. 33	96	88	85	99	105	102	107
11. v. 33	100	110	107	105	105	101	100
12. v. 33	93	111	82	105	105	116	112
13. v. 33	93	87	83	106	96	100	110
14. v. 33	84	86	91	105	100	105	108
15. v. 33	105	91	105	110	100	105	105
16. v. 33	88	91	88	110	100	98	116
17. v. 33	92	85	93	108	105	—	112
22. v. 33	88	84	88	110	116	—	105
23. v. 33	98	93	92	113	117	—	109
24. v. 33	95	110	108	100	105	—	105
25. v. 33	90	108	105	105	108	—	110
26. v. 33	88	88	91	105	116	—	116
27. v. 33	95	81	92	116	105	—	107

10. v. 33 Nos. 1 and 6 injected with 3·5 ml. brain extract.
Nos. 2, 3, 4 and 5 injected with 3·5 ml. anterior lobe extract.

14. v. 33 Nos. 1, 3 and 7 injected with 2·0 ml. anterior lobe extract.
Nos. 2 and 4 injected with 2·0 ml. brain extract.

23. v. 33 Nos. 2, 3, 4 and 5 injected with 4·0 ml. anterior lobe extract.
Nos. 1 and 7 injected with 4·0 ml. brain extract.

As in the same experiment with males, injection of anterior lobe extract into normal rabbits caused an increased excretion of creatinine, while injection into operated animals had no effect on the high creatinine output. Brain extract had no effect either in the normal or in the ovariectomised animals.

Discussion.

The results of ovariectomy and of injection of ovarian extracts affords evidence of the existence of an endocrine relationship between the ovaries and creatinine metabolism. The fact that both ovarian and testicular extracts have the same effect on the high creatinine level of ovariectomised animals suggests that the active principle in the ovarian extract is similar to, if not identical with, the active principle in the testicular extract.

Evidence presented in a previous paper [Schrire and Zwarenstein, 1933] suggests that the persistent increase in creatinine which occurs as the result of

castration is due primarily to hypertrophy of the anterior lobe of the pituitary. The results recorded above (Table V) indicate that the post-ovariectomy increase in creatinine is referable to the same factor.

Two outstanding differences emerge on comparing the effect of gonadectomy on creatinine excretion in the two sexes. The post-castration rise in creatinine commences 3 to 4 months after removal of the testes and in some cases sooner. In females the creatinine level is still normal 5 months after ovariectomy. It is unfortunate that owing to unavoidable circumstances no determinations could be carried out during the 6th–8th post-operative months. When determinations could again be resumed in the 9th post-operative month a definite increase in creatinine output was apparent.

In the ovariectomised animals the absolute individual rises in creatinine output above the values for the pre-periods were of the order of 41 %. Parallel normal controls showed an increase of about 25 % so that the true increase due to ovariectomy was actually about 16 %. Castration of males on the other hand led to a 25–50 % increase in the output of creatinine. Since in both sexes the effect on creatinine is primarily due to a pituitary hypertrophy, this difference is explicable on the assumption that functional hypertrophy of the anterior lobe is more pronounced in males than in females. This suggestion is supported by the observations of Hatai [1913] who found that the pituitary of castrated male rats was about 70 % heavier than that of spayed females, and by the results of Evans and Simpson [1929] who showed that the anterior lobe of the pituitary of gonadectomised male rats was more potent than that of gonadectomised females, as judged by the capacity of implants to produce precocious oestral changes in young females.

The excretion of creatine was too variable to allow of any certain conclusion regarding the effect of ovariectomy.

Summary.

1. Ovariectomy of adult female rabbits leads to a 16 % increase in the excretion of urinary creatinine commencing at least 5 months after the operation. The latent period is longer and the increase smaller than in castrated males.

2. Injection of saline suspension of ovary causes a fall in the high urinary creatinine of ovariectomy. Both ovarian and testicular extracts cause a slight fall when injected into normal animals. Larger and significant decreases occur on injection into ovariectomised animals.

3. Injection of extract of the anterior lobe of the pituitary leads to an increased excretion of urinary creatinine in normal females but has no effect on the high creatinine output of ovariectomised animals.

4. The results reported above afford evidence of an endocrine relationship between the ovaries and creatinine metabolism, this relationship being an indirect one through the anterior lobe of the pituitary.

REFERENCES.

Bellerby (1933). *Biochem. J.* **27**, 118.
Evans and Simpson (1929). *Amer. J. Physiol.* **89**, 375.
Hatai (1913). *J. Exp. Zool.* **15**, 297.
McGee, Juhn and Domm (1928). *Amer. J. Physiol.* **87**, 406.
Moore, Price and Gallagher (1930). *Amer. J. Anat.* **45**, 71.
Schrire and Zwarenstein (1932, 1). *Biochem. J.* **26**, 118.
—— —— (1932, 2). *Biochem. J.* **26**, 1886.
—— —— (1933) *Biochem. J.* **27**, 1337.

XLVIII. THE RELATIONSHIP OF THE METABOLIC NITROGEN OF THE FAECES TO BODY WEIGHT AND TO FOOD INTAKE FOR RATS.

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In the computation of biological values of proteins, investigators are divided in the manner of estimating the so-called "metabolic nitrogen of the faeces." Thomas [1909] and Martin and Robison [1922] thought this loss of body nitrogen to be constant, irrespective of the quantity of food ingested. Mitchell [1924] has considered it to be proportional to the amount of food dry matter consumed. Boas Fixsen and Jackson [1932], who favour the former viewpoint, make the following statement: "Mitchell [1924] argued that the amount of faecal nitrogen of endogenous origin excreted by a given animal was proportional to the weight of dry food ingested and that this ratio varied for different rats. In our experience, however, the results of several series of experiments on the same rat receiving nitrogen-free diets provide no evidence in support of such a relationship, at any rate where adult rats are concerned. On the contrary, in our work the same rat has shown an excretion of faecal nitrogen which is roughly constant in amount, in spite of large variations in the food intake...."

As these observations of Boas Fixsen are not in harmony with those made at this laboratory, the literature has been searched for evidence bearing upon the problem. A summary was made of all rat metabolism data relating to low-nitrogen diets that are available. This summary includes 1160 determinations of metabolic nitrogen (about one-third of which is from the author's unpublished data and the remainder from the literature) with rats of different body weights and consuming different amounts of food. The correlation of metabolic nitrogen and food consumed secured from these data is shown in Fig. 1. The regression line has a significant slope, which measures the rate of increase in metabolic nitrogen as more food is consumed. The rectilinearity of these data throughout most of their range seems apparent. The means of the arrays of the data when grouped follow a straight line except at the lowest and highest food intakes; the significance of these exceptions will be discussed later.

It cannot be stated, however, that all of the metabolic nitrogen excreted is proportional to the food consumed, since the intercept of the regression line on the y axis (zero food intake) is a significantly positive value. This part of the metabolic nitrogen obviously is not related to food intake.

There is, therefore, a constant fraction of the metabolic nitrogen, as well as a fraction which varies with food intake. If for any reason one fraction is much larger than the other, the impression may easily be created that the metabolic nitrogen is all of that fraction, either all constant or all proportional to food intake. Mitchell [1924; 1926] noted a relationship of metabolic nitrogen to body

[1] National Research Council Fellow in the Biological Sciences, 1931–33.

weight, but recognised with his small rats a much greater relationship to food intake. Boas Fixsen with large rats found "no evidence in support of such a relationship."

The correlation coefficient (r_{FM}) between food intake and metabolic nitrogen obtained from these 1160 observations is $0{\cdot}745 \pm 0{\cdot}009$. Correlation coefficients much nearer unity have been obtained by the author with data from individual rats[1]. The correlation coefficient (r_{WM}) between body weight and metabolic nitrogen is $0{\cdot}502 \pm 0{\cdot}015$, demonstrating that the relationship of metabolic nitrogen to body weight with these data is much less than the relationship of metabolic nitrogen to food consumption. To express adequately the relationship of metabolic nitrogen to food intake or body weight, irrespective of any relationship that may exist between body weight and food intake, it is necessary to make use of partial correlation coefficients. The partial correlation coefficient ($r_{FM.W}$) of food intake and metabolic nitrogen independent of body weight is $0{\cdot}744 \pm 0{\cdot}009$, while that ($r_{WM.F}$) of body weight and metabolic nitrogen independent of food intake, is $0{\cdot}499 \pm 0{\cdot}015$, indicating, as with simple correlation, that a closer relationship exists between food intake and metabolic nitrogen than between body weight and metabolic nitrogen.

The fact that the metabolic nitrogen in the faeces is related to body weight as well as to food intake, suggests that the constant fraction of the metabolic nitrogen, represented by the intercept of the regression line on the metabolic nitrogen axis in Fig. 1, varies with the body weight of the rat. Evidence confirming this suggestion may be deduced from these data. At the upper right-hand corner of Fig. 1, it will be noticed that the metabolic nitrogen values associated with the greatest food intakes, which were secured only with the larger rats, are all located above the regression line. Likewise, the points which are above the regression line at its lower end, and cause the data to depart from rectilinearity, were obtained again with the larger rats. In fact, the values obtained from larger rats tend to be above the regression line and those obtained from small rats below the regression line throughout the scale of food intake, the regression line representing the food intake-metabolic nitrogen relationship for the rat of average weight. Over two-thirds of the data from rats above average weight are located above the regression line, while the reverse is true with rats smaller than average.

There are 43 determinations of faecal nitrogen at a daily food intake of 9 g. that merit special study. The points referring to the larger rats are above the others, showing again that the larger the rat the greater is the constant fraction of the metabolic nitrogen. These 43 determinations with constant food intake show a correlation (r_{WM}) of $0{\cdot}830 \pm 0{\cdot}032$ between body weights and metabolic nitrogen values. The tendency for larger rats to have greater metabolic nitrogen excretions at any one level of food intake is evidenced also by the significantly positive partial correlation coefficient ($r_{WM.F} = 0{\cdot}499 \pm 0{\cdot}015$) between body weight and metabolic nitrogen.

Thus, it may be considered that the larger the rat the larger the constant fraction of the metabolic nitrogen, and assuming the same slope one may imagine a number of lines parallel with the regression line in Fig. 1, each line representing metabolic nitrogen as a function of food intake for one rat, the height of the line

[1] From data to be published elsewhere, the correlation coefficients between the food intake and the metabolic nitrogen of the same rat fed at from 10 to 19 levels of food intake were as follows for six different rats: $0{\cdot}981 \pm 0{\cdot}006$, $0{\cdot}971 \pm 0{\cdot}009$, $0{\cdot}993 \pm 0{\cdot}003$, $0{\cdot}964 \pm 0{\cdot}011$, $0{\cdot}937 \pm 0{\cdot}019$ and $0{\cdot}924 \pm 0{\cdot}023$.

varying with the weight of the rat. Hence, the data of Boas Fixsen, resulting from experiments with large rats (which consumed less food relative to body weight than small growing animals) show comparatively less change in the total metabolic nitrogen when food intake is varied and thus do not reveal the effect of food consumption on the metabolic nitrogen to the same extent as Mitchell's data with smaller rats, excreting correspondingly less of the constant fraction of the metabolic nitrogen and relatively more of the variable fraction related to the intake of food.

Another reason why Boas Fixsen failed to detect a relationship between metabolic faecal nitrogen and food intake pertains to her experimental technique, which does not appear to be sufficiently exacting. Maintaining a constant level of food intake during an experimental period of 4 days does not ensure regular excretion. Unless the same intake is continued for several days before the

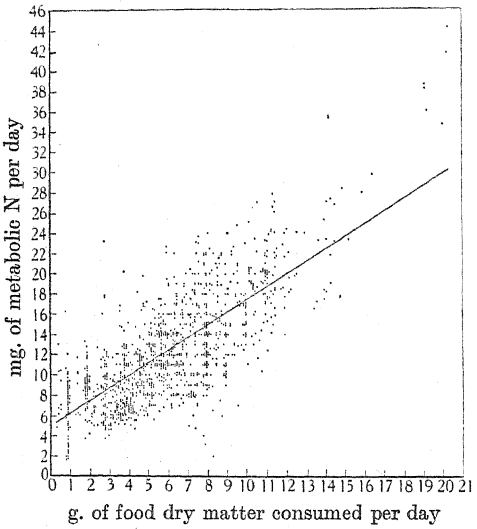

Fig. 1.

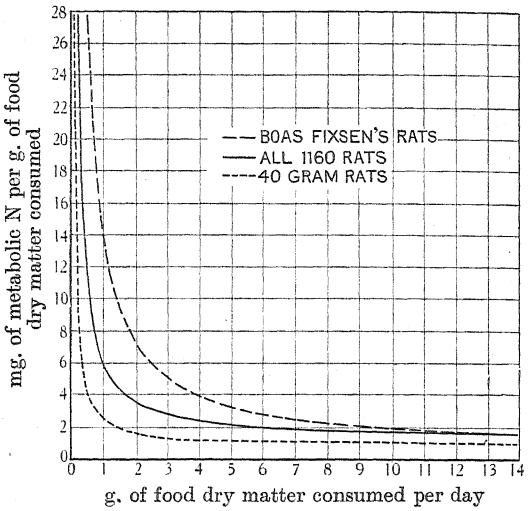

Fig. 2.

collection period, there is a marked tendency for the amount of faecal dry matter collected at extreme levels of food intake, large or small, to regress toward the mean, *i.e.* toward the amount of faeces from the usual intakes of food prevailing before the experimental collection period. Longer collection periods would also diminish errors of this type. The determination of faecal nitrogen reveals little of quantitative value unless the amount of faeces analysed is excreted as a result of exactly that food intake to which it is ascribed. One cannot be sure of obtaining a quantitatively accurate sample of faecal dry matter in four-day periods without feeding a faeces marker. Distinct demarcation of faeces can be obtained by using alternately ferric oxide and chromic oxide to colour the first feeding of successive periods. Also, nitrogen-free diets, such as have been used by Boas Fixsen [1930], having little indigestible substance, do not favour regular defaecation. Furthermore, the slope of the regression line of metabolic nitrogen on food intake for such highly digestible diets is less than that of the regression line for those having more indigestible dry matter. Thus, the metabolic nitrogen of Boas Fixsen's rats increased on an average only 0·56 mg. per g. of dry food

consumed, while the average for all rats considered was an increase of 1·25 mg. of metabolic nitrogen per g. of food dry matter consumed.

Considering these factors of technique, involving the use of large rats, inaccurate demarcation of faeces, no preliminary periods[1], short collection periods and highly digestible diets, it is not surprising that the total metabolic nitrogen as measured should appear "roughly constant" for any one rat. Nevertheless, an examination of Fig. 1 reveals that the data of Boas Fixsen (indicated by circles) harmonise fairly well with the other 1103 observations. In fact, all of her data show a correlation coefficient between food intake and metabolic nitrogen of $0{\cdot}481 \pm 0{\cdot}069$, indicating a significant relationship for the pooled data which is scarcely suggested by the data from the individual rats which were fed at eight or fewer levels of food intake.

As a part of the metabolic nitrogen is constant and not proportional to food intake, it might be reasoned that to estimate the metabolic nitrogen in a period of protein feeding a regression equation (instead of the ratio introduced by Mitchell [1924]) should be derived from two levels of feeding of low-nitrogen food. The curves in Fig. 2[2] show the ratio "mg. of metabolic nitrogen per g. of food dry matter consumed" as a function of food intake. The ratio can be considered to be fairly constant and within the limits of experimental error for all but the lowest food intakes. The solid curve (derived from the data in Fig. 1) is for the "average" rat of the population. With larger rats the curve is to the right and above, its slope extending to greater food intakes. This indicates, of course, that with larger rats greater food intakes are required before the ratio becomes practically constant. The data from smaller rats produce a curve which is to the left and below, reaching a level at lower food intakes. Each curve approaches an asymptote for food intakes infinitely large, that is not influenced by the size of the rat. All curves approach infinity as the food intake approaches zero.

An increase in the amount of indigestible substance in the diet raises the position of the curve, but does not change its form. Hence, in determinations of the biological values of proteins it is important that the dry matter of the standardising low-nitrogen diet be of approximately the same digestibility as that of the protein-containing ration. The curves reveal that the ratio "mg. of metabolic nitrogen per g. of food," although not the most exact statement of the mathematical relationship between food intake and metabolic nitrogen, in practice lies well within the limits of experimental error when more than a certain minimum quantity of low-nitrogen food is ingested daily, and that it affords an appropriate means of computing the metabolic nitrogen in protein-feeding periods.

Conclusions.

The metabolic nitrogen in the faeces of rats consists of two fractions, one related to the body weight and the other to the intake of dry food.

The ratio of metabolic faecal nitrogen to the intake of dry food assumes a practically constant level for intakes of food above a certain minimum value, which seems to approximate to that required for maintenance of body weight in the case of the usual low-nitrogen diets used in protein evaluation work. Hence, in

[1] [Note by Editor.] The author is mistaken on this point [see Boas Fixsen, 1930, p. 1796].

[2] Considering that the regression line in Fig. 1 is described by the equation $y = a + bx$ in which y is the metabolic nitrogen, a the y-intercept, b the slope constant and x the food intake, the curves in Fig. 2 were fitted by the equation $y = \frac{a}{x} + b$, y being the ratio of metabolic nitrogen to food intake in the latter case, the other symbols having the same significance.

determinations of the biological values of proteins by Mitchell's technique, the ratio can logically be used in estimating the metabolic nitrogen in the faeces in the protein-feeding periods, if its limitations are born in mind. Furthermore, no better method of estimation appears to be available.

REFERENCES.

Boas Fixsen (1930). *Biochem. J.* **24**, 1794.
—— and Jackson (1932). *Biochem. J.* **26**, 1919.
Martin and Robison (1922). *Biochem. J.* **16**, 407.
Mitchell (1924). *J. Biol. Chem.* **58**, 873.
—— (1926). *Nat. Res. Counc. Bul.* **55**.
Thomas (1909). *Arch. Anat. Physiol.* **219**.

XLIX. THE INFLUENCE OF ARSENATE AND ARSENITE ON THE ENZYMIC BREAKDOWN OF PHOSPHORIC ACID ESTERS.

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The experiments of Harden and Young [1906; 1911] showing that arsenate and arsenite ions accelerate the rate of liberation of inorganic phosphate from hexosediphosphate by the enzymes of yeast juice have led to much speculation as to the mechanism involved. Recent investigations of the effects of arsenate on the activity of the phosphatases and the glycolytic enzyme systems present in different kinds of biological material have yielded results which have been variously interpreted. All but the most recent of this work has been summarised by Harden [1932].

The view, originally expressed by Harden and Young, that the stimulating effect is exerted on the hexosephosphatase of yeast juice is open to criticism as the result of investigations in several laboratories. The experiments of Meyerhof [1927] with yeast and muscle, of Macfarlane [1930] and of Harden [1933] with yeast, and of Braunstein [1931] with rabbit erythrocytes support the conception that the effect is exercised not on the phosphatase but on the glycolytic system. This hypothesis is not considered by Morgulis and Pinto [1932] to be generally applicable in view of their results with rabbit and dog blood. Recently Braunstein and Lewitow [1932] have observed progressive diminution of the inorganic arsenate in mixtures of yeast, sugar, arsenate, toluene and water and they suggest that the accelerating effect of arsenate on fermentation and glycolysis depends on the formation of a labile hexosearsenate.

The present paper represents a study of the effects of arsenate and arsenite ions on the enzymic breakdown of phosphoric acid esters. The work was undertaken with the purpose of determining whether stimulation of phosphatase activity by these ions can be regarded as a general phenomenon or whether their effects on the rate of breakdown of phosphoric acid esters concern rather the mechanisms by which the organic portions of the esters are degraded by the accompanying glycolytic or similar enzymes. The materials chosen as the source of enzyme included four bacterial species, *Clostridium acetobutylicum* Weizmann, *Propionibacterium jensenii* van Niel, *Bacillus lactis aerogenes* and *Bacillus subtilis*, in addition to takadiastase, dried yeast, pig kidney and ox erythrocytes. Both sodium hexosediphosphate and sodium glycerophosphate were employed as substrates since the restricted use of the former in the investigation of mixtures containing both phosphatase and glycolytic systems might conceivably lead to erroneous conclusions. Furthermore, since the early work of Buchner [1903] and of Harden and Young [1911] showed that arsenite also accelerates the rate of fermentation by yeast juice, examination of the influence of this ion has been included in the present study. The method described by Pett [1933] for the

determination of inorganic phosphate in the presence of arsenate permits greater accuracy in the investigation of the effects of this ion in a wide range of concentrations than was hitherto possible.

Experimental.

Enzyme preparations.

Bacteria. Cl. acetobutylicum and *P. jensenii*, in the dried form, were prepared in the manner previously described [Pett and Wynne, 1933, 1]. *B. lactis aerogenes* was grown for 4 days in a sterilised medium containing 3 % glucose and 0·6 % peptone in diluted yeast extract. The organisms were centrifuged, washed once, dried and ground. *B. subtilis*, grown for 6 days in a similar medium, was treated in the same manner. The bacteria were used in the proportion of 0·1 g. dried organisms per 10 ml. enzyme digest.

Yeast. S. cerevisiae, isolated from baker's yeast, was grown in a medium containing 5 % glucose, 1 % peptone and suitable inorganic salts dissolved in diluted yeast extract. After 5 days the organisms were centrifuged, washed, dried and ground as usual; 0·05 g. dried cells was used in 10 ml. of digestion mixture. An extract of dried yeast was prepared by shaking 5 g. of the powder with 100 ml. of chloroform-saturated water for 24 hours. The mixture was then centrifuged and filtered; 1 ml. of the clear filtrate was used in 10 ml. digest.

Takaphosphatase. 20 g. of commercial takadiastase were suspended in 50 ml. of water, chloroform was added and the mixture was shaken for 15 hours. A small insoluble residue was removed; the solution contained a large amount of free phosphate which was eliminated by adding the equivalent amount of $MgCl_2$ and dilute NH_4OH until precipitation was just complete. Of the filtrate from this precipitate 1 ml. was used in 10 ml. of enzyme digest.

Mammalian kidney[1]. Fresh pig kidney was ground with twice its weight of water and allowed to stand for several hours. The mixture was filtered, and the filtrate was adjusted to p_H 6·0; after standing for 15 hours the solution was again filtered. The filtrate was adjusted to neutrality and stored in a refrigerator; 0·5 ml. of this solution was used in 10 ml. of digestion mixture.

Ox erythrocytes. The oxalated blood was treated in the manner described by Roche [1931]. Two ml. of the haemolysed solution were added to 0·5 ml. of buffer, 0·5 ml. of solution of arsenate or arsenite and 2 ml. of 2·5 % sodium glycerophosphate or of 1·25 % sodium hexosediphosphate.

Substrates.

The sodium glycerophosphate employed was obtained from The Eastman Kodak Company and consisted very largely of the β-ester. Sodium hexosediphosphate was prepared from the commercial calcium salt; the final preparation contained a small amount of monophosphate and a trace of inorganic phosphate.

General conditions.

The p_H optima of the phosphatases investigated are shown in Table I. The optimum for the yeast enzyme acting on hexosephosphate was reported by Kertesz [1930], for takaphosphatase on glycerophosphate by Kay and Lee [1931] and for kidney-phosphatase by Kay [1926]. The optima for the enzymes of the first two species of bacteria listed in the table have been reported by the present

[1] The authors are grateful to Mr G. A. McVicar for a supply of kidney-phosphatase prepared in this manner.

authors [1933, 1]. All the other recorded optima were determined as a part of the present investigation.

Table I. *Showing the* p_H *optima of various phosphatases.*

Enzyme source	Substrate: Hexosephosphate	Substrate: Glycerophosphate
Cl. acetobutylicum	6·0	5·1
P. jensenii	6·0	7·0
B. lactis aerogenes	5·8	5·5
B. subtilis	6·8	10·8
Sacc. cerevisiae	6·3	4·0
Takadiastase	4·2	4·0
Mammalian kidney	8·9	8·9
Ox blood cells	6·5	6·1

Potassium hydrogen phthalate buffers were used in the acid range and glycine buffers in the alkaline range; at p_H 7·0 no buffer was used. All solutions were adjusted to the desired p_H before mixing and the p_H of each mixture was determined after hydrolysis. No change greater than 0·3 p_H was observed.

Solutions of Na_3AsO_4 and of Na_2HAsO_3 having the following concentrations were prepared: M, $M/4$, $M/10$, $M/25$, $M/50$, $M/75$, $M/100$ and $M/200$. Of these solutions 1 ml. portions were added to 9 ml. of digestion mixture.

The total volume in each case was 10 ml., made up of 4 ml. of substrate (2·5 % glycerophosphate or 1·2 % hexosediphosphate), 4 ml. of buffer, 1 ml. of enzyme if liquid or 1 ml. of water together with the previously indicated weight of dried organisms, and 1 ml. of arsenate or arsenite solution. When other solutions, such as NaF, were added simultaneously simple adjustments of the volume of buffer were made, the concentration being kept constant. The mixtures were rocked in L-shaped tubes in a water-bath at 37·5°. At zero time and after 1 hour 2 ml. samples were added to 10 ml. portions of 5 % trichloroacetic acid solution. The precipitates were centrifuged and 10 ml. portions of the clear liquids were placed in 25 ml. volumetric flasks. If arsenate were present reduction was then effected in the manner described by Pett [1933]. Inorganic phosphate was determined by Fiske and Subbarow's method, using 3N acid; in several experiments duplicate results were obtained with Briggs's modification of the Bell and Doisy method.

Control tubes containing (*a*) enzyme, added ions and buffer, (*b*) substrate, added ions and buffer were incubated with the experimental tubes. The amount of inorganic P in the latter at zero time was subtracted from the 1-hour values, and from the figures so obtained was subtracted the sum of the increases in inorganic P in the two control tubes. Other tubes containing enzyme, buffer and substrate but no arsenic were included in all experiments. To the net amount of inorganic P (after allowing for the P released in suitable controls) liberated in such a digest containing a given enzyme preparation was assigned a value of 100. All the other results obtained with the same enzyme were compared with this standard value. The results are expressed in Table II.

Effect of fluoride.

In every instance of acceleration by arsenate or arsenite the acceleration could be lessened or removed by NaF in suitable concentration. This observation is complicated by the fact that fluoride retards the phosphatase activity of certain cells, such as yeast [Lipmann, 1928] and muscle [Lipmann, 1929]. However, fluoride up to $M/100$ concentration does not affect the hexosephosphatase

Table II. *Effects of arsenate and arsenite on the activity of various phosphatase preparations on glycerophosphate and hexosephosphate.*

Figures give the percentage hydrolysis under the influence of ions shown, as compared with the hydrolysis without added ions.

Conc. of ion in digest.	*Cl. acetobutylicum*		*P. jensenii*		*B. lactis aerogenes*		*B. subtilis*		Takadiastase		Yeast: Dried		Yeast: Aqueous extract		Pig kidney		Ox erythrocytes	
	Glycero-phosphate	Hexose-phosphate	Glycero-phosphate	Hexose-phosphate	Glycero-phosphate	Hexose-phosphate	Glycero-phosphate	Hexose-phosphate	Glycero-phosphate	Hexose-phosphate	Glycero-phosphate	Hexose-phosphate	Glycero-phosphate	Hexose-phosphate	Glycero-phosphate	Hexose-phosphate	Glycero-phosphate	Hexose phosphate
Arsenite:																		
M/2000	100	100	**100**	**100**	97	99	99	97	98	**113**	100	**100**	100	100	100	100	102	**108**
M/1000	100	100	**115**	**107**	90	97	98	96	101	**143**	100	**100**	100	100	100	100	100	**120**
M/750	101	100	**118**	**113**	—	—	97	94	—	—	100	—	—	—	100	103	—	—
M/500	100	97	**114**	**117**	87	96	95	91	100	**158**	100	**108**	100	100	100	102	100	**132**
M/250	98	95	**112**	**106**	84	94	95	88	103	**158**	95	**119**	100	98	97	105	100	**135**
M/100	90	93	**90**	**95**	76	93	95	83	103	**151**	94	**110**	95	96	95	106	91	**114**
M/40	—	—	—	—	—	—	—	—	97	**113**	92	**98**	90	—	—	—	90	**93**
M/10	—	—	—	—	—	—	—	—	—	—	—	—	—	95	92	92	—	—
Arsenate:																		
M/2000	100	100	**98**	**108**	101	99	93	98	100	**97**	98	**98**	100	100	98	100	103	**101**
M/1000	80	98	**117**	**122**	86	100	90	84	91	**141**	79	**115**	98	100	81	96	96	**115**
M/750	75	99	**123**	**132**	—	90	88	83	—	—	—	—	—	—	—	—	—	—
M/500	78	95	**116**	**136**	82	89	87	79	60	**167**	62	**161**	80	96	54	54	84	**130**
M/250	67	87	**92**	**207**	76	81	83	75	45	**162**	55	**153**	63	96	48	28	73	**144**
M/100	68	71	**49**	**265**	50	69	82	74	23	**155**	38	**132**	58	97	33	17	73	**188**
M/40	—	—	—	**306**	—	—	—	—	—	—	—	—	43	—	—	10	62	**170**
M/10	—	31	—	**305**	—	—	—	—	15	**106**	25	**124**	34	100	12	0	—	—
M	—	—	—	**9**	—	—	—	—	—	—	—	—	—	—	—	0	—	—

activity of *P. jensenii* [Pett and Wynne, 1933, 1]. This system is therefore well adapted for the investigation of the relation of fluoride to arsenate acceleration. Table III shows the results of an experiment of this kind.

Table III. *Diminution of the accelerating effect of arsenate by fluoride.*

Arsenate	Fluoride	mg. P released in 10 ml.
none	none	0·542
M/100	none	1·381
none	*M*/40	0·530
M/100	*M*/1000	1·368
M/100	*M*/400	1·287
M/100	*M*/200	1·207
M/100	*M*/100	1·123
M/100	*M*/40	0·873

The series can be carried no farther than *M*/40 NaF because higher concentrations affect the phosphate determination. The process used in reducing the arsenate has no effect on the recovery of phosphate in the presence of fluoride. It is evident that fluoride definitely reduces the arsenate acceleration.

A different type of experiment is of interest in this connection. The phosphorus reagents were added to a flask containing 2 ml. *M*/500 arsenate and to another containing 2 ml. *M*/500 arsenate and 2 ml. *M*/100 fluoride. After standing for 1 hour at 50° the flask containing fluoride developed only about 75 % of the intensity of colour in the other flask. Na_3AsO_4 does not yield a precipitate with NaF, even in a solution saturated with respect to both, so that the method by which arsenate is removed from action is not one of precipitation.

It was considered possible that the Na ions in the NaF solution appreciably depressed the dissociation of the Na_3AsO_4, but experiments with NaCl and Na_2SO_4 indicated that up to *M*/40 concentration these salts could not depress the arsenate acceleration to an extent equivalent to more than one-quarter of the fluoride effect.

While these facts do not fully explain the fluoride action they suggest that the fluoride ion forms a "complex ion" with the arsenate ion. Arsenate is thus converted into an ineffective form.

Discussion.

The results recorded in Table II lead to several observations. (*a*) The liberation of inorganic phosphate by the various enzyme preparations was not uniformly affected by arsenate and arsenite ions. The release of phosphate was accelerated in some instances, unaffected in others and definitely retarded in the remainder. If it be assumed that the phosphatases of the various preparations are identical in their mode of action on both substrates, an assumption which is not yet free from difficulties, the enzymes might be expected to respond similarly to the influence of arsenate and arsenite. Since they do not, it can be concluded that the influence of these ions is exercised not on the phosphatase but on some other mechanism which by its operation liberates inorganic phosphate. (*b*) In cases in which arsenate accelerated the reaction, arsenite had a similar though usually less marked effect; the optimum concentrations of the two ions were not always identical. (*c*) No stimulation occurred with an aqueous extract of dried yeast though the activity of the dried yeast preparation itself was stimulated by both arsenite and arsenate. (*d*) Acceleration with glycerophosphate as substrate was observed only with the *P. jensenii* preparation.

It is probable that all the enzyme preparations possessed in addition to phosphatase a glycolytic mechanism capable of breaking down, at least partially, the hexose portion of hexosediphosphate. However, in only four of the nine cases in which the influence of arsenate on the degradation of this ester was studied was there any evidence of acceleration. It is apparent therefore that, in addition to having no consistent effect on the hydrolytic activity of the various phosphatases, the arsenate ion has no universally demonstrable accelerating influence on the activity of the glycolytic enzyme systems. This assumes that, under the conditions of the experiments, accelerated breakdown of the organic component of the ester would lead incidentally to accelerated accumulation of free phosphate.

The increase in inorganic phosphate as the result of the action of yeast, kidney, takadiastase, erythrocyte and *Cl. acetobutylicum* preparations on glycerophosphate can be regarded as proof of the presence of a true phosphatase because it is probable that none of these preparations is able to attack the glycerol portion of the ester. But no stimulation by arsenate or arsenite occurred with any of these glycerophosphatases. On the other hand all the other bacteria used can metabolise glycerol; it has been shown, furthermore, that *P. jensenii* [Pett and Wynne, 1933, 2] and *B. lactis aerogenes* (unpublished work of the authors) can, after drying, partially break down the glycerol residue of the ester. However, in the case of only one organism, *P. jensenii*, did arsenate or arsenite exert any accelerating influence on the rate of glycerophosphate breakdown.

A satisfactory interpretation of these results is not yet possible. Although it can be concluded that arsenates and arsenites do not accelerate the activity of phosphatases in general, the lack of uniformity of the effects of these ions on the activity of the various preparations, as measured by phosphate liberation, is somewhat confusing. The evidence supports the hypothesis that any observed accelerating effect is due to a stimulation of the enzyme system responsible for the degradation of the organic part of the ester. Furthermore the specificity observed suggests that the influence is exercised at some particular stage of this breakdown, common to some but not all of the preparations used. Further speculation must be supported by more complete experimental evidence.

In this connection it is of interest to observe that, although the aqueous extract of dried yeast possessed an active phosphatase which was not stimulated by arsenate or arsenite, the dried yeast itself responded to the influence of both ions. When tested in a 1 % solution of sodium hexosediphosphate in micro-fermentation tubes the yeast extract failed to cause the liberation of gas. It is evident that the complete zymase system was not present in the extract: possibly this deficiency was reflected in the failure of arsenate and arsenite to cause stimulation.

The experiments using cytolysed ox erythrocytes are of interest in view of the recent findings of Morgulis and Pinto [1932] and of Braunstein [1931]. The latter was unable to demonstrate arsenate stimulation of the phosphatase activity of rabbit serum and erythrocytes on hexosediphosphate, but in experiments in which the esters of the cells constituted the substrates for the accompanying enzymes he obtained results which were interpreted as indicating activation of the glycolytic mechanism by arsenate. On the other hand Morgulis and Pinto [1932] do not regard such stimulation of glycolytic systems as a general phenomenon because concentrations of arsenate which definitely accelerated the rate of glycolysis in mixtures containing rabbit blood preparations caused retardation of glycolysis using dog blood; in all cases the liberation of inorganic phosphates was accelerated. They therefore concluded that the increased rate of phosphate liberation in blood-glycolysing systems under the

influence of arsenate is due to a stimulation of the blood-phosphatases. In the case of ox erythrocytes, in the present work, arsenate acceleration of the rate of liberation of inorganic phosphate was observed with hexosediphosphate as substrate but not with glycerophosphate. If the stimulation were exercised on the phosphatase mechanism one would have expected acceleration of the hydrolysis of both substrates to occur. The evidence strongly suggests that in the case of ox erythrocytes the arsenate effect is exercised on the glycolytic system rather than on the phosphatase.

SUMMARY.

The rate of liberation of inorganic phosphate from glycerophosphate and hexosediphosphate through the action of several enzyme preparations was studied in relation to the influence of varying concentrations of arsenate and arsenite ions. Preparations of the following were employed: *Cl. acetobutylicum*, *P. jensenii*, *B. subtilis*, *B. lactis aerogenes*, yeast, takadiastase, pig kidney and ox erythrocytes.

No evidence was obtained to show that the activity of phosphatase systems in general is accelerated by arsenate or arsenite. The observations support the hypothesis that the accelerated enzymic liberation of phosphate under the influence of these ions is due to an effect exercised at some stage of the breakdown of the organic portion of the phosphoric acid ester.

The diminution of the stimulating effect of arsenate by fluoride was investigated and the suggestion is advanced that this may be due to the formation of a complex ion, thus rendering a portion of the arsenate ineffective.

REFERENCES.

Braunstein (1931). *Biochem. Z.* **240**, 68.
—— Lewitow (1932). *Biochem. Z.* **252**, 56.
Buchner (1903). Zymasegärung, p. 187. (Oldenbourg, Munich and Berlin.)
Harden (1932). Alcoholic fermentation, 4th edition. (Longmans, Green and Co., London.)
—— (1933). *Biochem. Z.* **258**, 65.
—— and Young (1906). *Proc. Chem. Soc.* **22**, 283.
—— —— (1911). *Proc. Roy. Soc. Lond.* B **83**, 451.
Kay (1926). *Biochem. J.* **20**, 791.
—— and Lee (1931). *J. Biol. Chem.* **91**, 135.
Kertesz (1930). *J. Amer. Chem. Soc.* **52**, 4117.
Lipmann (1928). *Biochem. Z.* **196**, 3.
—— (1929). *Biochem. Z.* **206**, 171.
Macfarlane (1930). *Biochem. J.* **24**, 1051.
Meyerhof (1927). *Biochem. Z.* **183**, 176.
Morgulis and Pinto (1932). *J. Biol. Chem.* **95**, 621.
Pett (1933). *Biochem. J.* **27**, 1672.
—— and Wynne (1933, 1). *Biochem. J.* **27**, 1660.
—— —— (1933, 2). *Trans. Roy. Soc. Can.* (in press).
Roche (1931). *Biochem. J.* **25**, 1724.

L. EFFECT OF ULTRA-VIOLET RADIATION AND HEAT UPON PROTEIN SOLUTIONS OF LOW CONCENTRATIONS.

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Protein solutions of acid or alkaline reaction show a marked increase of absorptive power in the ultra-violet spectrum after prolonged irradiation with the light of a quartz mercury lamp [Spiegel-Adolf and Krumpel, 1927]. The shape of the absorption curve is not altered if the irradiation be carried out in ordinary air, the irradiated product in this case behaving like the original protein at higher concentration [Hausmann and Spiegel-Adolf, 1927; Spiegel-Adolf, 1928]. The protein irradiated under the same conditions but without addition of electrolytes coagulates spontaneously nearly completely, as has been shown in a previous paper [Spiegel-Adolf, 1927]. Becker and Szendrö [1931] working on highly diluted protein solutions (1·5–2·5 mg./100 ml.) found on the other hand that irradiation, especially in presence of oxygen, seemed to destroy the proteins. The degradation products though showing an increased absorption for ultra-violet radiation had lost the absorption band characteristic for proteins and their normal derivates. In order to explain this apparent discrepancy new experiments were started on protein solutions of different concentrations.

In a first series of investigations measurements of the refraction have been undertaken in order to detect traces of cleavage of the protein irradiated under conditions comparable with those employed by Spiegel-Adolf and Krumpel [1927]. Boiled samples of uncoagulable protein mixtures were studied similarly, since Spiegel-Adolf and Krumpel [1929] have shown that such boiled protein solutions retain their original absorptive power. Changes of the refraction of proteins after denaturation by heat and strong acid have been reported by Obermayer and Pick [1905].

As in the proposed experiments little or no heat change was to be expected and only the effects of heat and light of short wave-length were to be compared, an interferometer (Zeiss-Löwe) was used. Since the interferometric value of a 0·1–0·05 % pseudoglobulin solution attained a stable value after 2 hours of irradiation with the light of a quartz mercury lamp (Hanovia, 220 v. d.c.) and the protein had at the same time practically disappeared from the solution, the irradiation time was limited in this series to 2 hours, the distance of 40 cm. making cooling unnecessary. The protein solutions used were pseudoglobulin and serum-albumin separated from horse serum by fractionation with $(NH_4)_2SO_4$, purified repeatedly by refractionation, dialysis and electrodialysis. The stock solution of the pseudoglobulin was 5 %, the c_H was $1{\cdot}40 \times 10^{-7}$, the conductance $\kappa = 1{\cdot}11 \times 10^{-5}$. The 2 % solution of serum-albumin had c_H $1{\cdot}17 \times 10^{-5}$, a conductance $\kappa = 9 \times 10^{-6}$. The amounts of HCl or KOH added to the protein solutions were within the range of those used in the previous communications.

As in former experiments the heating of the solutions in a water-bath under reflux was limited to 15 minutes. The results are summarised in Table I A and B.

The figures are interferometric units, a 20 mm. cell being used.

Table I.

A. Serum-albumin.

(Final concentration in all samples 0·2 %.)

		Native	Boiled	Irradiated	Boiled and irradiated	Irradiated and boiled
0·001 *N* KOH	Obs.	325	333	331	326	331
	Calc.	330				
0·005 *N* KOH	Obs.	375	379	379	380	410
	Calc.	381				
0·001 *N* HCl	Obs.	320	322	326	329	350
	Calc.	326				
0·005 *N* HCl	Obs.	356	356	356	382	393
	Calc.	362				

B. Pseudoglobulin.

(Final concentration in all samples 0·1 %.)

0·001 *N* KOH	Obs.	196	198	199	203	199
	Calc.	201				
0·005 *N* KOH	Obs.	245	249	245	248	249
	Calc.	252				
0·001 *N* HCl	Obs.	194	196	199	196	203
	Calc.	198				
0·005 *N* HCl	Obs.	226	231	228	231	231
	Calc.	235				

Additions of acid or alkali to serum-albumin and pseudoglobulin give lower values than those calculated. These findings agree with those of Thomas and Mayer [1928], whose interpretation seems applicable to all kinds of proteins. The changes in refraction after irradiating or boiling are negligible; they do not exceed 3 % of the interferometric value of the native protein but seem to be outside the limit of error of the method. The influence of heating is most marked in alkaline solutions, *i.e.* under conditions favouring maximum hydrolysis, while irradiation shows its effects most clearly in an acid medium. Nevertheless in most cases there are no differences between the effects of heat and irradiation and when such differences do occur they are so small as to give no support to the suggestions of Becker and Szendrö [1931]. When heating precedes the irradiation the changes if any are much smaller [*cf.* Spiegel-Adolf, 1929].

In a further series of experiments the behaviour of proteins submitted to heat or irradiation at various concentrations without additions of electrolytes was studied. The irradiation and the heating were done in the manner already described, the fluids after filtration through Schleicher and Schüll filter-paper No. 590 were tested for their refraction, protein-content and gold sol reaction.

The results are summarised in Table II A and B.

These results show that both kinds of denaturation are sufficient to precipitate the total protein; only in the most concentrated samples can traces of the latter be detected after irradiation for 2 hours at 40 cm. distance; these disappear on further exposure. Though especially in the highest degree of dilution denaturation seemed to cause but a faint opacity of the solutions it was always possible to get a clear filtrate by using the above-mentioned filter.

Interferometric measurements of the filtrates indicate that not all refractive material has been precipitated with the protein. This may mean that the interferometric method is more sensitive in detecting protein than flocculation by

Table II.

A. Serum-albumin.

Concentration %		Heat	IU	SSSR	GSR	Irradiation	IU	SSSR	GSR
0·16	*a*		307	+	+		307	+	+
	p	Coagulation	19	−	−	Coagulation	37	trace	+ + +
0·08	*a*		144	+	+		144	+	+
	p	Coagulation	13	−	−	Coagulation	22	−	+ + +
0·04	*a*		73	+	+		73	+	+
	p	Coagulation	7	−	−	Coagulation	21	−	+ + +
0·02	*a*				−				
	p				+				
0·016	*a*		27	+	−		27	+	−
	p	Coagulation	4	−	+	Coagulation	13	−	+ + +
0·004	*a*				−				
	p				−				
0·0032	*a*		6	+	−		6	+	−
	p	Opalescence but filtrable	5	−	−	Clear	8	−	+ + +

B. Pseudoglobulin.

0·2	*a*		374	+	+		374	+ +	+
	p	Coagulation	20	−	− R_V	Precipitation	44	trace	+
0·1	*a*		193	+	+		193	+	+
							28	−	+
	p	Coagulation	8	−	+	Precipitation	43	−	+
0·05	*a*		91	+	+		91	+	+
	p	Coagulation	5	−	+	Precipitation	42	trace	+
0·02	*a*		48	+	+		48	+	+
	p	Coagulation	4	−	−	Precipitation	19	−	−
0·004	*a*		10	+	+		10	+	+
	p	Coagulation	0	−	−	Distinct opalescence	8	−	+
0·002	*a*		5	+	−		6	trace	−
	p	Coagulation	2	−	−	Doubtful opalescence	7	−	−

IU = Interferometric units in 20 mm. cell.
a = before; *p* = after.
SSSR = Reaction with sulphosalicylic acid.
GSR = Reaction with colloidal gold.
R_V = Red with trace of violet.

sulphosalicylic acid or that precipitation of the protein is accompanied by some cleavage, or finally that the solutions contain something else beside protein. It does not seem possible to exclude any of these possibilities altogether. But the fact that only in the case of heated proteins does the refraction of the filtrates decrease practically to zero with increasing dilution, while in the case of irradiation it reaches finally the refraction value of the original solution, seems to indicate that irradiation of proteins causes not only denaturation but also cleavage. This supposition agrees with the changes in odour and colour.

Further evidence of the cleavage of proteins submitted to irradiation is afforded by the gold sol reaction. This is more sensitive for pseudoglobulin than for serum-albumin but does not reach the sensitiveness of the sulphosalicylic acid test. Colloidal gold, however, can be used besides for the classification of proteoses, and, according to the explanation of the clinical gold sol tests, is able to reveal pathological changes in proteins where other methods fail.

Table II A and B shows that in the cases of heated proteins with the exception of serum-albumin in a range between 0·02 and 0·01 % the filtrates do not show any gold sol reactions. This confirms the results of Ogiu and Pauli [1932], who made similar observations, and the fact that the filtrates were practically free from protein. But the filtrates of irradiated proteins even when they show no trace of proteins with sulphosalicylic acid give a strong gold sol reaction. The type of the gold sol reaction is somewhat different from the two types of gold sol flocculation by proteins described by Ogiu and Pauli [1932], the change in the present case being directly from pink to blue and white.

The finding of a substance giving a positive gold sol reaction seems to confirm the results of Becker and Szendrö [1931] as to the partial splitting of the protein by irradiation. But the formation of this particular proteose depends upon the intensity and perhaps upon the wave-length of the radiation—the protein solutions themselves acting as selective filters, whose absorption is maximum between 292·7 and 261 $m\mu$. If for instance a 0·2 % serum-albumin solution is not filtered after the first hour of irradiation when it is nearly opaque but is irradiated for another hour, then the sulphosalicylic acid test becomes negative, but the gold sol reaction becomes negative too. If a solution of 0·5 % pseudoglobulin is irradiated in the usual way, after 4 hours it reaches a stage at which the sulphosalicylic acid test is still positive, but the gold sol reaction due to the protein content of the solution has already disappeared. Two more hours of irradiation cause total disappearance of the protein reaction, while the gold sol reaction becomes strongly positive. Nevertheless the solid content of the filtrate is only 1/10 of the initial protein content, and the end absorption starting at 230 $m\mu$ cannot account for the increased absorption of irradiated protein solutions containing acid or alkali within a range of 400–267·5 $m\mu$.

Finally experiments were performed under conditions similar to those used by Spiegel-Adolf and Krumpel [1927]. 0·5 % solutions of serum-albumin and pseudoglobulin containing 0·005N HCl or KOH were irradiated during 5½–6 hours at a distance of 30–40 cm. from the light source. The solutions were protected against evaporation by cork stoppers fitting tightly into the quartz test-tubes. (Controls of pure protein solutions were used in order to ascertain the effect of irradiation upon them.) After the exposure the solutions were exactly neutralised. They still contained traces of heat-coagulable proteins proving the existence of small amounts of undenatured protein. After renewed filtration the solutions showed a slight reaction with sulphosalicylic acid but no gold sol reaction. This was taken to show that the effect of irradiation depends greatly on the original protein content of the solution and on the amount of acid or alkali, as these determine the initial protein concentration through the entire experiment. The different kinds of cleavage caused in protein solutions of varying concentration or electrolyte content by irradiation may explain the discrepancy of the spectrographic findings of different authors which have been the starting-

Table III.

	Filtration	Period of irradiation hrs.	Electrolytes	Gold sol reaction
0·2 % serum-albumin	Filtration after 1st hour	2	None	+
0·2 % serum-albumin	No filtration	2	None	–
0·5 % pseudoglobulin	Filtration	4	None	–
0·5 % pseudoglobulin	Filtration	6	None	+
0·5 % serum-albumin or pseudoglobulin	Filtration	5½–6	0·005N HCl or KOH	–(after neutralisation)

point of this paper. (The above-mentioned experiments are summarised in Table III.)

Becker and Szendrö [1931] have made elaborate investigations of the nitrogen content and certain chemical properties of the diluted protein solutions after irradiation. It seemed desirable to extend the investigation in a more physico-chemical direction, particularly concerning the relations to other proteoses of the substance giving a positive gold sol reaction.

In order to get solutions of this substance of higher concentrations 0·1 % solutions of serum-albumin and pseudoglobulin were irradiated in flat dishes at 15 cm. distance directly under the tube of the quartz mercury arc lamp; the surface of the dish was about 50 cm.^{2}, the fluid layer having a depth of 1 cm. After 6 hours of irradiation more than half of the total protein content, *i.e.* 0·026–0·03 g. of the initial 0·05 g. was precipitated; the fluid was then concentrated to 1/10 vol. on the boiling water-bath.

The solution was clear with a marked yellow tinge, the solid content was in several samples between 0·21 and 0·27 %, the specific conductivity was $3{\cdot}53 \times 10^{-4}$ ohms^{-1}, the c_H $6{\cdot}22 \times 10^{-6}$. The latter was measured with a hydrogen electrode, the whole outfit having been carefully checked before and after the determination. The absorptive power in ultra-violet radiation was different from that of protein, being, for the same solid content, rather higher than those of serum-albumin or pseudoglobulin. Using 5 mm. quartz cells a 0·4 % solution of serum-albumin absorbed all light from wave-length 245 $m\mu$, while the absorption of the filtrate from the irradiated sample started approximately at 320 $m\mu$ (Table IV).

Table IV. *Extreme non-absorbed wave-lengths.*

Concentration %	Serum-albumin $m\mu$	Filtrate of irradiated serum-albumin $m\mu$	Proteose of Witte peptone $m\mu$
0·4	245	320	—
0·2	235	275	245
0·1	231	240	—

Pseudoglobulin shows a similar behaviour. But contrary to the findings of Spiegel-Adolf and Krumpel [1927] for concentrated protein solutions irradiated in the presence of acid or alkali, the filtrate did not give any special absorption band beside the end absorption, confirming in this respect the findings of Becker and Szendrö [1931].

The filtrate gave a positive biuret reaction with a slight red-violet tinge and positive reactions with Millon's reagent and trichloroacetic acid, while the test with sulphosalicylic acid was negative and no H_2S could be traced after heating with alkali. Alcohol causes a slight opalescence when its concentration reaches 80 %. The filtrate shows a slight opalescence when saturated with ammonium sulphate but no change on half saturation. If the test is made according to the method of Nonne-Appelt-Schumm for spinal fluid it is negative or doubtful.

The filtrate flocculates gold sol in concentrations equivalent approximately to 0·0005 mg. in 1 ml., the derivatives of serum-albumin and pseudoglobulin giving practically the same figures which are smaller than those given by the native proteins (Table V).

But while the flocculating power of the native proteins can be inhibited by the presence of neutral salts no such effect can be detected with the filtrates obtained by denaturation by radiation, indicating a difference in the nature of the flocculation.

Table V. *Gold sol reactions. The figures indicate concentrations* %.

	0·02	0·01	0·005	0·002	0·001	0·0005	0·00025
Serum-albumin	R_{vvv}	R_v	R	R	R	R	R
Pseudoglobulin	colourless	colourless	colourless	V	V	R_v	R
Filtrate from irradiated serum-albumin	colourless	colourless	colourless	V	R_{vvv}	R_{vv}	R_v

R = cherry red; V = violet; suffixes v = indicate shades of red with tinge of violet.

After dialysis and electrodialysis there is a nearly complete loss of the conductance, absorption and refractive power of the fluid which no longer gives the biuret reaction and shows only traces of its original gold sol-flocculating power.

Taking into consideration the properties of the filtrate here described it does not seem possible to identify the fraction or fractions with any known cleavage-product of proteins according to the classification of Pick [1925]. Nor does the gold sol reaction give any help. According to Zsigmondy [1920] both secondary proteoses and peptones are able to flocculate colloidal gold with blue colour without additional salt. Zunz [1906] has determinated in 13 different fractions of secondary proteoses and peptones the amount necessary to change the colour of red gold to violet. His most potent fraction (Albumose BI) has only 1/10 of the flocculating power of the filtrate obtained by irradiation. Zunz [1907] also stated that secondary proteoses are unable to flocculate mastic sol; the filtrate obtained by irradiation of proteins did precipitate mastic sol though in somewhat higher concentrations (Table VI).

Table VI. *Mastic sol reactions. The figures indicate concentrations* %.

	0·086	0·043	0·021	0·01	0·005	0·002	0·001	0·0005	0·0002
Serum-albumin	×	×	×	×	+	0	0	0	0
Pseudoglobulin	×	×	×	×	×	×	+	0	0
Filtrate from irradiated pseudoglobulin	×	× +	+	0	0	0	0	0	0

(× = flocculation, + = cloudiness.)

It is only possible to state that the filtrate from irradiated protein is practically free from proteins and contains some secondary proteoses and probably peptones. But considering the very low acidity of the filtrate it seems scarcely likely that the flocculation of colloidal gold is caused by peptones.

In order to ascertain whether the special kinds of proteoses found in the filtrates of irradiated proteins are missing entirely from those of heat-coagulated proteins a 0·2 % solution of pseudoglobulin was heated on the water-bath for 15 minutes. After removing the precipitated protein the filtrate was concentrated to 1/50 vol. The solid content of such a solution amounted to 0·11–0·13 %. Reactions with sulphosalicylic acid and trichloroacetic acid were positive while the gold sol reaction remained entirely negative.

This seems to indicate that in denaturation of proteins by heat and by light of short wave-length, differences can be detected not only in the precipitated fraction [Spiegel-Adolf, 1927] but also in the cleavage products. Changes in odour and colour had already suggested this.

The fact that some proteoses show such a high degree of flocculating power towards colloidal gold seems to be of some interest in connection with the behaviour of spinal fluids in certain pathological cases. Though the results of the reaction of Lange can nearly always be explained by the content of proteins in the fluid and by the ratio between albumin and globulin, nevertheless there are

cases such as the one referred to by Kyrle *et al.* [1920] in which there was a marked gold sol flocculation without an increase in proteins. Besides, Epstein and Rubinstein [1926], using the adsorption method of Willstätter, were able to show that the fractions giving gold sol curves characteristic for general paralysis did not show protein reactions. Though no identity between this substance and the one obtained through irradiation can be reasonably expected, experiments were started in which an equal amount of the latter was added to a spinal fluid and the gold sol reaction of this mixture compared with that of the spinal fluid alone.

The spinal fluids were sterile, Wassermann-negative and did not show any increase in proteins. As in all previous tests two kinds of gold sol were used simultaneously, one made according to the method of Bredig with consecutive purification by dialysis, the other by reduction with formaldehyde. The precipitation was made according to Lange. The results of such an experiment are given in Table VII.

Table VII. *Spinal fluid "K.M." containing equal amounts of 40 times concentrated filtrate from irradiated protein.*

Diluted ...	10	20	40	80	160	320
Red	.	.	.	.	.	.
Red-violet	.	.	.	.	+	+
Violet	.	.	.	+	.	.
Blue	.	.	.	.	.	.
Bluish white	+	+	+	.	.	.
White	.	.	.	.	.	.

The spinal fluid alone when diluted in the same way did not change the colour of the red gold sol.

These experiments show that the addition of proteoses derived from horse-serum by irradiation elicits in normal spinal fluid a behaviour towards colloidal gold practically identical with the reaction of the same fluid in cases of general paralysis. The test of Nonne-Appelt was negative. Nevertheless there seems to exist a protective power in normal body fluids against the gold sol-precipitating effect of the proteoses, as a much greater amount of the latter was necessary to obtain effects similar to those reached with watery dilutions of the proteoses alone. Solutions of pure serum-albumin or pseudoglobulin did not show this effect.

The behaviour of the proteoses obtained by irradiation seems to suggest the occurrence of other atypical proteoses in spinal fluids in pathological cases as an explanation of a positive gold sol test without increased protein content.

Summary.

1. Protein solutions of 0·1–0·2 % irradiated or heated in the presence of acid or alkali show practically unchanged refraction.

2. Electrolyte-free serum-albumin and pseudoglobulin in concentrations from 0·2 to 0·002 % are practically entirely coagulable by heat or irradiation; in the latter case, only the filtrate shows an increase in refraction and a positive reaction with colloidal gold.

3. Protein solutions of 0·2–0·5 % containing acid or alkali do not show, after irradiation, neutralisation and heat-coagulation, positive reactions with colloidal gold.

4. Filtrates of protein coagulated by heating or irradiation were concentrated 10–50 times and investigated physico-chemically. The results seem to indicate the presence of atypical proteoses in the latter case.

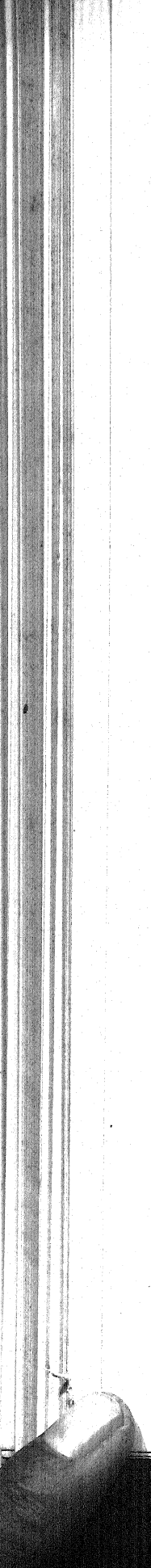
5. Evidence is given that the proteoses are distinguished by strong light absorption. Their power to flocculate colloidal gold exceeds the power of genuine proteins 10–40 times. They also precipitate mastic sol.

When a concentrated solution of the proteoses obtained by irradiation of electrolyte-free proteins is added to normal spinal fluid the mixture gives a gold sol reaction similar to that obtained in cases of general paralysis.

The author wishes to express her thanks to Dr Temple Fay for kindly providing spinal fluids.

REFERENCES.

Becker and Szendrö (1931). *Pflüger's Arch.* **228**, 755.
Epstein and Rubinstein (1926). *Kolloid Z.* **40**, 307.
Hausmann and Spiegel-Adolf (1927). *Klin. Woch.* **6**, 2182.
Kyrle, Brandt and Mras (1920). *Wien. klin. Woch.* **33**, 1745.
Obermayer and Pick (1905). *Beitr. chem. Physiol. Path.* **7**, 331.
Ogiu and Pauli (1932). *Biochem. Z.* **250**, 535.
Pick *cit.* Kestner, Chemie der Eiweisskörper (1925). 4th edit. (Vieweg, Braunschweig.)
Spiegel-Adolf (1927). *Biochem. Z.* **186**, 181.
—— (1928). *Biochem. Z.* **197**, 197.
—— (1929). *Biochem. Z.* **213**, 475.
Spiegel-Adolf and Krumpel (1927). *Biochem. Z.* **290**, 28.
—— —— (1929). *Biochem. Z.* **208**, 45.
Thomas and Mayer (1928). *Proc. Soc. Exp. Biol. Med.* **25**, 667.
Zsigmondy (1920). Kolloidchemie. 3rd edit. (Leipzig.)
Zunz (1906). *Bull. Soc. Roy. Sci. Med. Nat.* **64**, 174.
—— (1907). *Arch. Internat. Physiol.* **5**, 245.



LI. A NOTE ON THE REDUCTION POTENTIAL OF CYSTEINE-CYSTINE MIXTURES.

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GHOSH *et al.* [1932] have shown that in solution of $p_H > 7$, the electrolytic reduction of cystine to cysteine at a mercury electrode sets up a reversible potential system at the electrode. The potential of the system was determined by the concentration of the oxidant and the reductant and agreed with the thermodynamic formula

$$-E_h = E_0 - \frac{RT}{F} p_H - \frac{RT}{F} \log \frac{[\text{Cysteine}]}{\sqrt{[\text{Cystine}]}} \qquad \ldots\ldots(1),$$

where $E_0 = +\cdot 079$.

More recently Green [1933] has shown that the evidence brought forward by Ghosh *et al.* could be substantiated if the potential were measured at the particular mercury surface at which the cystine was reduced. But if the potential were measured at a new mercury surface, introduced into the system by an auxiliary electrode, a difference of about 60 mv. was at once observed and the potential at the auxiliary mercury electrode conformed to Dixon and Quastel's [1923] equation

$$E = E_0 - \frac{RT}{F} p_H - \frac{RT}{F} \log [\text{Cysteine}] \qquad \ldots\ldots(2).$$

Quoting Barron *et al.* [1929] on the possibility of complex formation between cysteine and mercury in absence of oxygen when a galvanic cell is set up, Green confirmed the observation of Ghosh *et al.* that after electroreduction of cystine, H_2S did not give a precipitate, but he did not agree with their conclusion that no complex formation occurred in their experiments, and suggested that such a complex was indeed formed but was confined to the mercury surface.

It is however well known that a mercury surface when exposed to air is covered with an adsorption layer. Reeser and Sissing [1922] found the thickness of the adsorbed layer to be between 22 and 34 Å. McBain [1932] believes this layer to be a film of mercuric oxide, as it is not affected by evacuation for eight hours at room temperature using a Gaede pump.

The present authors in their previous work were guided by the suspicion that a film of adsorbed oxygen or oxide on the surface of the mercury electrode was responsible for the results of Dixon and of Barron *et al.* This film could not be removed by bubbling an inert gas like nitrogen, but could only be destroyed by a cathodic reduction process at the surface. The experimental method of Green with slight modifications is well suited to test directly the accuracy of this hypothesis.

Chemically pure N_2 gas prepared from NH_4Cl and $NaNO_2$ was dispersed into minute bubbles by means of a Jena gas filter and passed up a tower containing about 500 cm. length of a solution of alkaline pyrogallol prepared according to

the method of Haldane and Makgill [1933]. The complete absorption of all traces of oxygen was confirmed by passing the gas over a piece of white phosphorus which did not glow, showing that the oxygen pressure was less than 0·0007 mm.

The cell consisted of two chambers A and B connected by a stopcock T with 8 mm. bore. The inner portion of each chamber consisted of two tubes one within the other. The outer tube served as the nitrogen inlet and the inner tube as the siphon for contact with the calomel electrode. The outer limb of the siphon was filled with agar saturated with KCl. A platinum wire fused into the bottom of the vessel made electrical contact with the mercury electrode.

The chamber A was first filled with a buffer solution having the same p_H as the cystine solution. The bore of the stopcock T was also filled with buffer solution. The stopcock was then closed and the chamber B filled up with the buffered cystine solution. The apparatus was then set up as depicted in Fig. 1

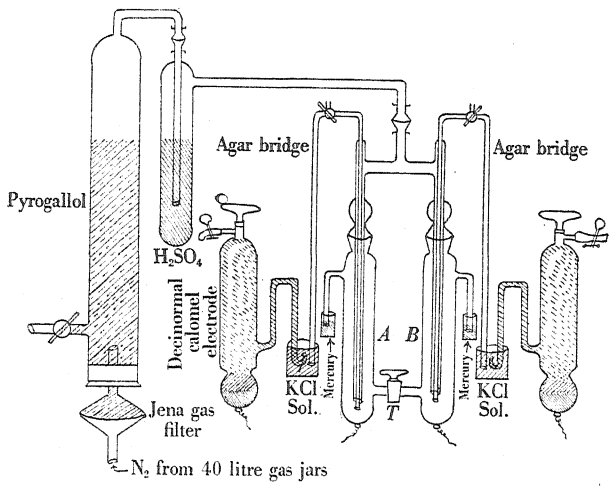

Fig. 1.

and the chambers de-aerated by a vigorous current of nitrogen. A current of 5 milliamperes was passed for 5–10 minutes between the mercury in the chamber B as cathode and a platinum anode in the KCl solution beyond the agar bridge. The mercury in the chamber A was similarly subjected to cathodic depolarisation for 3–4 hours. It is essential for the success of the experiment that the potential drop at the cathode in chamber A shall be sufficient to discharge hydrogen gas but much less than is necessary to discharge the alkali metal ions which were used to prepare the buffer solutions. The results become erratic if alkali metal amalgams are formed at the surface. Hence cathodic depolarisation for sufficiently long periods with very low currents is necessary. The stopcock T was now opened; by variation of the height of the mercury columns at the nitrogen outlets from the vessel as well as by passing a vigorous current of nitrogen for 3–4 hours, the solutions in both the chambers were well mixed. The potentials at the mercury surfaces in each chamber were then measured against a decinormal calomel electrode.

The results are given in Table I.

The potentials of the mercury surfaces in chambers A and B become ultimately the same and are given by the equation (1).

Table I.

p_H	Conc. cystine	Conc. cysteine	E_h in mv. Chamber A	E_h in mv. Chamber B	E_0 calc. according to (1)
7·00	0·00171 M	0·00099 M	−232	−230	0·082
7·50	—	—	−243	−242	—
8·00	—	—	−307	−308	—
8·50	—	—	−384	−383	—

The experimental results recorded above therefore confirm the hypothesis that an oxygen or oxide film on the mercury surface is responsible for setting up a potential in cystine-cysteine mixtures which follows the equation (2). Elimination of this film and complete removal of oxygen from the solution give rise to potentials which follow the thermodynamic equation (1).

It is well known that in presence of oxygen, complex formation of mercury with —SH compounds takes place rapidly. It is also probable that when a very limited supply of oxygen is available, as is the case in an adsorbed film of oxygen or oxide, mercury forms complexes with —SH compounds in preference to —S—S— compounds. The system Hg—HgS—H_2S gives potentials [Immerwahr, 1901] which would obviously depend only on the concentration of H_2S. The authors are of opinion that the Dixon potentials may be due to a similar system, where HgS has been replaced by a compound formed by interaction of the —SH compound with the oxide film at the mercury surface and H_2S replaced by sulphydryl compounds.

REFERENCES.

Barron, Flexner and Michaelis (1929). *J. Biol. Chem.* **81**, 743.
Dixon and Quastel (1923). *J. Chem. Soc.* **123**, 2943.
Ghosh, Roy Chowdhury and Ganguli (1932). *J. Ind. Chem. Soc.* **9**, 43.
Green (1933). *Biochem. J.* **28**, 678.
Haldane and Makgill (1933). *Analyst*, **58**, 378.
Immerwahr (1901). *Z. Elektrochem.* **7**, 477.
McBain (1932). Sorption of gases and vapours by solids, 272. (London.)
Reeser and Sissing (1922). *Proc. Acad. Sci. Amsterdam*, **24**, 108.

LII. STUDIES ON THE KINETICS OF HAEMOLYTIC SYSTEMS.

V. THE LYTIC EFFECT OF MIXTURES OF TWO LYSINS.

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THE only existing paper on this subject is one by Sen and Mitra [1929], in which a study of the lytic effects of mixtures of saponin, sodium taurocholate, sodium glycocholate and sodium oleate, in various proportions, led to the conclusion that the effects of two lysins acting together are probably additive in most cases, although in some instances the effect of the mixture may be greater than the sum of the individual effects. Sen and Mitra's experiments, however, are hardly conclusive, for two reasons. (1) No account is taken of the p_H changes which occur when two lysins such as saponin and sodium taurocholate are mixed together, which may result in either an acceleration or an inhibition of the action of one or both lysins. Sen and Mitra recognise this themselves. (2) The existing data are not presented in such a way as to be susceptible of exact analysis, and the conclusion that the effects of two lysins are additive in most cases really rests on nothing more substantial than an impression.

This paper is concerned with a re-investigation of the problem, and, as will be seen, leads to conclusions somewhat more definite than those of Sen and Mitra.

1. *Methods.*

Except in the case of systems containing sodium oleate, all the lysins were dissolved in an isotonic phosphate buffer at p_H 6·77, this buffer being selected because at this p_H the time-dilution curves for sodium taurocholate and sodium glycocholate are of the usual regular form and devoid of the "zones" so characteristic of these lysins in systems of certain p_H. In the case of systems containing sodium oleate the buffer used was a phosphate buffer at p_H 8·5, for this lysin is not sufficiently active in buffers of lower p_H. The cell suspension was a "standard" suspension consisting of the thrice washed cells of human blood finally suspended in 20 ml. of 0·9 % NaCl for every ml. of blood used. It is well to let the lysins stand for some hours before they are used, for as Sen and Mitra remark, their lytic activity often decreases with time.

To illustrate the procedure, consider the case in which the combined effects of saponin and sodium taurocholate are to be investigated quantitatively. First we plot a time-dilution curve at 25° for the saponin, the systems consisting of 0·8 ml. of various dilutions of the lysin (1 in 10,000, 1 in 20,000, *etc.*), 0·8 ml. of buffer, and 0·4 ml. of the standard suspension. Let the quantity of lysin present in each system be c_1 and the time for complete lysis t_1. Next we take a system consisting of 0·8 ml. of taurocholate, 1 in 2000 (say), 0·8 ml. of buffer and 0·4 ml. of suspension and find the time taken by it to show complete lysis. Suppose that it is the

same time as that taken by a dilution of 1 in 60,000 saponin ($33{\cdot}3\gamma$ in the system of total volume 2 ml.). Then 1 in 2000 taurocholate is equivalent to 1 in 60,000 saponin, or 1000γ of taurocholate to $33{\cdot}3\gamma$ of saponin, equivalence being judged by the taking of equal times to produce complete lysis. Call this equivalent quantity of saponin c_3.

Now prepare systems containing both lysins. Each contains 0·8 ml. of the 1 in 2000 taurocholate, 0·8 ml. of various dilutions of saponin, and 0·4 ml. of cell suspension. Let the time for complete lysis be t_2. Referring to the time-dilution curve for saponin acting alone, we find t_2 corresponding to a quantity c_2; then, if the effects are additive, one would expect to find

$$c_3+c_1=c_2,$$

c_1 being the initial quantity of saponin present in the system containing the mixed lysins which haemolyses in time t_2, c_2 the quantity of saponin which, acting alone, would produce lysis in time t_2, and c_3 having the meaning defined above. I say that one would expect to find this relation, because it seems to follow so obviously from the reasoning, but, as a matter of fact, I shall show that the relation would be true under special conditions only, and that these are far from obvious.

If (c_3+c_1) be not equal to c_2, the activity of the mixture appears to be depressed below, or increased above, the sum of the activities of the two lysins by the factor $c_2/(c_3+c_1)$, and the value of this ratio can be used to measure the extent of the departure from a simple additive effect.

2. *Results.*

1. *Saponin and digitonin.* These lysins are both glucosides and have haemolytic properties which are similar in almost every respect. When mixed, however, their combined effect is much less than their individual effects added together, as can be seen from the values for the ratio $c_2/(c_3+c_1)$ which are given in Table I.

Table I.

Saponin γ	Digitonin γ 40	25	18
100	0·89	0·53	0·59
50	0·90	0·52	0·55
40	0·92	0·56	0·59
25	0·87	0·60	0·54
20	0·83	0·57	0·51

In the case of these two lysins, the inhibition is greater when the lysins are present in the smaller concentrations, but, as will be seen below, this is not an invariable rule. I may remark that the second decimal of the value given for the ratio is probably not very reliable.

2. *Saponin and sodium taurocholate.* These two lysins differ quite considerably in their haemolytic properties, but as in the case of saponin and digitonin, there is mutual inhibition.

Table II.

Saponin γ	Taurocholate γ 2000	1000	667
100	0·73	0·98	0·98
67	0·71	1·00	0·97
40	0·72	0·89	0·93
25	0·84	0·85	0·86
20	0·89	0·88	0·80

As in Table I there is a tendency for the inhibition to increase as the quantity of saponin becomes less, but it does not change in a similar way as the quantity of taurocholate is varied. The greatest inhibition of all, indeed, is met with when the bile salt is present in the greatest concentration.

3. *Saponin and sodium oleate.* These two lysins are quite unlike in many respects, and the activity of a mixture of the two is far greater than that which would result from summing the individual activities.

Table III.

Saponin γ	Oleate γ 200	100	67
100	1·74	1·63	1·41
67	1·52	1·67	1·40
50	1·39	1·60	1·37
40	1·34	1·50	1·26
33	1·16	1·37	1·05
25	1·09	1·11	1·03

The acceleration in this case is greatest when the lysin concentration is greatest and falls off as either the quantity of saponin or of oleate present in the system is reduced.

4. *Sodium taurocholate and sodium glycocholate.* In the case of these two similar haemolysins, we again find more than an additive effect, and again the acceleration is greatest when the lysins are present in greatest quantity.

Table IV.

Taurocholate γ	Glycocholate γ 1000	800	670
1330	2·14	1·44	1·27
1000	1·81	1·36	1·20
800	1·39	1·33	1·16
667	1·33	1·29	1·11
500	1·20	1·18	1·07
400	1·12	1·09	1·04

5. *Sodium taurocholate and sodium oleate.* This combination of lysins gives the most curious results of all:

Table V.

Taurocholate γ	Oleate γ 200	100	67
2000	0·62	0·78	0·76
1000	1·02	0·70	0·79
500	1·54	1·07	0·89
400	1·70	1·20	0·93

Here we find more than an additive effect, or less than an additive effect, depending on the concentrations of the haemolysins present in the system.

Discussion.

At first sight these results appear to dispose of Sen and Mitra's contention that the total effect of mixtures of lysins is "in most cases probably additive," for the ratio $c_2/(c_3+c_1)$ is generally greater or less than unity. Before drawing such a

conclusion, however, we must carefully scrutinise the principles on which the investigation is based.

To simplify matters, let us first assume that both lysins, which we shall refer to as A and B, act on the same cell component. Existing theory states that lysis is complete when $x\gamma$ of lysin are used up in combining with a proportional quantity of cell component. Suppose that this occurs when lysin A transforms the component, and when $x=25$. Further, suppose that complete lysis takes time t_1 when A is used. When B is used, let the time be t_1 also: then we would say that the quantity c_2 of B has the same activity as the quantity c_1 of A, for both give complete lysis in the same time t. If the two lysins were acting together in these same concentrations, we should at first sight expect the time taken for complete lysis to be the same as that taken by (c_1+c_1) of A, if A were acting alone.

But a little consideration will show that this is only so if the value of n in the expression for the fundamental reaction for A and B is the same, *i.e.* the kinetics of the reaction between the cell component and A and between the cell component and B must both be described by the expression

$$kt=\frac{p}{p-1}\left\{c^{\frac{p-1}{p}}-(c-x)^{\frac{p-1}{p}}\right\},$$

with the same value of $p=1/n$. Or, in words, if two lysins produce complete haemolysis by transforming the same amount of cell component in the same time, they will transform different amounts of cell component in shorter times, unless the value of n is the same for the time-dilution curves of both lysins. This is a very important point, and is related to another point which has been discussed elsewhere [Ponder, 1932], *viz.* that if we have a quantity of lysin c_1 which, as the result of the presence of some inhibitor, appears to have been reduced to the smaller quantity c_2, then the quantity of lysin rendered inert by the inhibitor is (c_1-c_2) only if the value of n is the same in the presence and in the absence of the inhibitor.

Let us take a concrete example, in which 100γ of lysin A (*e.g.* saponin) act on cells to produce complete lysis when $x=25$. Suppose that $n=1{\cdot}0$ and $1/k=10$. The relation between t and the quantity of component transformed is then shown in Fig. 1, curve A, and the time for complete lysis is 1·23 minutes. Now let 100γ of B act, and suppose that $n=2{\cdot}0$ and $1/k=0{\cdot}59\times10^{-3}$. Curve B of the same figure shows the relation between t and x, and again complete lysis occurs in 1·23 minutes. In these concentrations the two lysins are accordingly equal to each other, at least when the time for complete lysis is used as a criterion. But if the transformation of $12{\cdot}5\gamma$ is taken as a criterion, they are unequal, for A transforms this quantity in 0·57 minute and B in 0·51 minute. And similarly for the transformation of any quantity less than 25γ.

Now suppose that we are ignorant of this effect of the different values of n, and mix 100γ of A with 100γ of B. Then we again get complete lysis when 25γ of lysin are transformed, and referring to the A-curve (arbitrarily used as a standard) we expect this to occur in 0·57 minute, for then if n were 1·0 for both A and B, we should get a transformation of $12{\cdot}5\gamma$ of A and $12{\cdot}5\gamma$ of B. In fact, we get 25γ used up in combining with the cell component in 0·54 minute as the result of 12γ of A being used up, and 13γ of B. This looks like an acceleration, and referring to the time-dilution curve for A, we find $c=200\gamma$ giving complete lysis in 0·57 minute and $c=210\gamma$ giving complete lysis in 0·54 minute. In such a case we should find the ratio $c_2/(c_3+c_1)$ to be 1·05, although in fact the effects of the two lysins were strictly additive.

This apparent acceleration is not very spectacular, but it becomes far greater if we use a smaller quantity of lysin, *e.g.* 35γ of A and $42{\cdot}5\gamma$ of B[1]. These both produce lysis in 5·44 minutes if the values of n and of $1/k$ are the same as the above. If n were the same for A and B, and the values of $1/k$ so adjusted as to give $t=5{\cdot}44$ for $x=25$, then we should have 25γ transformed in 1·9 minutes in a system containing both A and B in the above concentrations. In fact, when $n=2{\cdot}0$ for B and 1·0 for A, we get complete lysis in 1·25 minutes, for at this value

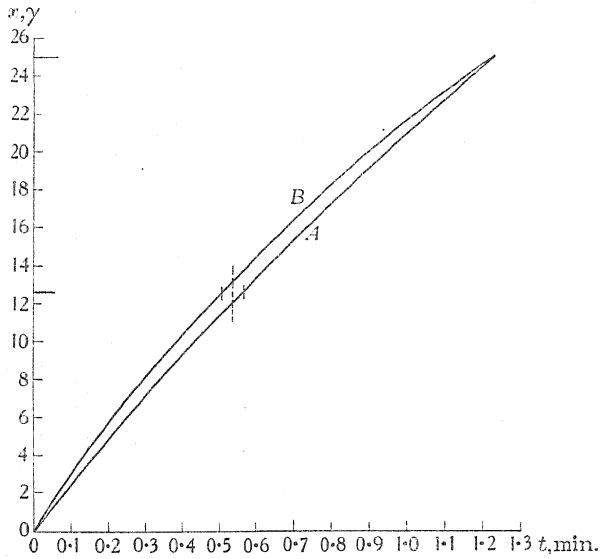

Fig. 1. Two curves showing the relation between time and the quantity of lysin transformed, A, when $n=1{\cdot}0$, B, when $n=2{\cdot}0$. For details, see text.

of t we have $8{\cdot}25\gamma$ of A transformed and $16{\cdot}75\gamma$ of B, the total being 25γ. Referring to the time-dilution curve for A, we get $t=1{\cdot}25$ when $c=99\gamma$ and $t=1{\cdot}9$ when $c=70\gamma$. So the ratio in this case turns out to be 1·4, which represents quite a considerable acceleration, although the activities of the two lysins are still strictly additive.

By the same reasoning, if we select as the standard curve that for the lysin with the higher value of n, instead of that for the lysin with the lower value of n, as above, the apparent accelerations become replaced by apparent inhibitions of the same magnitude.

It would be possible, at the expense of a rather unwarrantable amount of trouble, to investigate each one of the ratios shown in the preceding tables and to find out if the observed departures of the $c_2/(c_3+c_1)$ ratios from unity are such as could be accounted for by one of these "pseudo-accelerations" or "pseudo-inhibitions," but a conclusion may be arrived at in a quicker way, for under all circumstances the pseudo-acceleration or pseudo-inhibition is more obvious in the systems containing the more dilute lysins. We can accordingly draw up a

[1] If two concentrations of two lysins produce complete lysis in the same time, the same fractions of these concentrations produce lysis in different times, unless n is the same for both. This is so obvious that it requires no discussion.

Table showing what we should expect to find if the departures of the ratio from unity were the result of such pseudo-accelerations and inhibitions, and also what we find in fact.

Table VI.

Standard lysin	n	Second lysin	n	Result predicted	Result found	Increase or decrease in dilute system
Saponin	1·8	Digitonin	2·5	Accel.	Inhib.	Increase
Saponin	1·8	Taurocholate	1·6	Inhib.	Inhib.	Increase
Saponin	1·8	Oleate	1·3	Inhib.	Accel.	Decrease
Taurocholate	1·6	Glycocholate	1·2	Inhib.	Accel.	Decrease
Taurocholate	1·6	Oleate	1·3	Inhib.	Both accel. & inhib.	

It is therefore quite clear that even if all allowance is made for the occurrence of these pseudo-accelerations and inhibitions, we have no evidence that the activities of these haemolysins are in fact additive. Indeed, if we were to go through the labour of making the necessary corrections, the departure of the ratio from unity would be increased in as many cases as it would be decreased.

We are thus forced to one of two conclusions: either (*a*) that the effects of two lysins are not additive, or (*b*) that the additive effect is obscured by the lysins reacting with one another in such a way as mutually to inhibit or enhance each other's activities. This they might do by reacting with each other in solution, as two colloids very frequently do, or by interfering with each other in the region of the red cell surface. The two possibilities (*a*) and (*b*) are not mutually exclusive, but, as I shall show, the second is far the more likely.

Let us see what the hypothesis that the activities of two lysins are not additive amounts to, the term additive being used in the sense of this paper, and all inhibitory and acceleratory effects of the lysins on each other being either excluded or allowed for. Under such circumstances, the effects of the lysins must be additive if they affect the same cell component. If they affect two different cell components, their effects will not be additive unless we imagine that the result of partial destruction of two different and spatially separated components is equal, in an additive sense, to the much greater destruction of a single component. There is no precedent for such an idea, which is highly improbable in itself: in fact, if the two lysins attack different cell components, their effects will almost certainly not be additive. The hypothesis that the activities are not additive is therefore tantamount to a hypothesis that the lysins react with different components and demands that they shall not react with the same component. All the available evidence, however, indicates that different lysins react with the same cell component, probably the protein "stromatin" [see Ponder *et al.*, 1930], and they certainly all react with that component which is responsible for the discoidal form [Ponder, 1934]. We have accordingly no collateral evidence supporting possibility (*a*), and, while we cannot altogether disprove it, we can conclude that it is not a very likely one.

We have therefore to fall back upon the second explanation for the results obtained in the above tables, *viz.* that the additive effects of two lysins are usually obscured by various inhibitory and acceleratory phenomena which result from an interaction of the lysins with each other.

Summary.

When two simple lysins (saponin, digitonin, sodium taurocholate, sodium glycocholate or sodium oleate) act together in a system, the total activity is rarely the same as the sum of the two individual haemolytic activities. The most

likely explanation of the result is that the lysins interact with each other, thereby mutually enhancing or inhibiting each other's lytic activity.

Some curious difficulties arise in the analysis of the combined effects of two lysins, and these are discussed at some length.

REFERENCES.

Ponder (1932). *Proc. Roy. Soc. Lond.* B **110**, 1.

—— (1934). The mammalian red cell and the properties of haemolytic systems, Chapter 3. (Protoplasma Monographs. In the Press.)

—— Saslow and Yeager (1930). *Biochem. J.* **24**, 805.

Sen and Mitra (1929). *J. Ind. Chem. Soc.* **6**, 111.

LIII. THE HYDROLYSIS OF CERTAIN POLYSACCHARIDES AND PROTEINS BY THE ENDOSPORES OF AEROBIC BACILLI.

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A study of the enzymic content of the endospores of some twelve unidentified aerobic bacilli was made by Ruehle [1923]. He found that the spore preparations studied by him liquefied gelatin which contained 0·5 % of phenol and was covered by toluene, and this observation was confirmed by Cook [1931]. Ruehle also investigated the ability of certain of his preparations to hydrolyse fats and caseinogen and obtained negative, or at the best feebly positive, results. In a previous paper [Tarr, 1932] it was shown that almost every cell in a given culture forms spores when the aerobic bacillus being studied is cultivated under appropriate conditions. Later [Tarr, 1933] it was found that endospores of aerobic bacilli possess active respiratory catalysts which are quite different from the respiratory catalysts of the vegetative cells from which they are formed. In the present communication it is shown that endospores of aerobic bacilli possess an amylase and proteases.

Experimental.

The cultures employed (*B. subtilis* I; *B. subtilis* II and *B. mesentericus* I) were identical with those used in previous work [Tarr, 1932]. Endospore suspensions were prepared by the method previously described [Tarr, 1933] and were heated to 80° for 30 minutes immediately prior to use.

The amylase of bacterial endospores.

The presence of an amylase in bacterial endospores was verified by studying the hydrolysis of both starch and glycogen in the presence of toluene as antiseptic in the following manner. 250 ml. Erlenmeyer flasks received 50 ml. of 0·5 % solution of soluble starch (or glycogen), 10 ml. of $M/5$ phosphate buffer p_H 7·3, 15 ml. of water and 50 ml. of endospore suspension. The liquid in each flask was covered with 10 ml. of toluene, and the flask was corked and then incubated at 37°, shaking well each day. At suitable intervals portions of the solutions were removed for examination, the hydrolysis being followed in three ways: (1) the colour formed when a drop of the solution was mixed with a drop of $N/100$ iodine was noted; (2) the relative ability of 1 ml. of the experimental solution to reduce a drop of Benedict's solution was determined; (3) the amount of aldose present in a 5 ml. sample was estimated by the method of Willstätter and Schudel [1918], the results being reported in ml. of $N/100$ iodine per 5 ml. of solution.

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The results of this experiment which are recorded in Tables I and II show that heated spores of the organisms studied hydrolyse both starch and glycogen in the presence of toluene, for the typical reaction of the polysaccharide with iodine disappears in each case, the amount of reducing sugar increases, and the aldose titre increases. That the reducing aldose formed in each case is maltose and not glucose is shown by the following experiment.

Table I. *Hydrolysis of starch by bacterial endospores.*

Time in days	Iodine test			Reducing sugar test			Aldose formed (ml. N/100 I)		
	B. subtilis I	*B. subtilis* II	*B. mesentericus*	*B. subtilis* I	*B. subtilis* II	*B. mesentericus*	*B. subtilis* I	*B. subtilis* II	*B. mesentericus*
0	Blue	Blue	Blue	−	−	−	0·28	0·29	0·29
							0·29	0·29	0·29
2	Red-brown	Blue	Red-brown	+	+	−	—	—	—
3	Red-brown	Red-brown	Neg.	+	++	−	—	—	—
5	Red-brown	Neg.	Neg.	++	+++	+	0·54	0·53	0·42
							0·56	0·53	0·42
9	Neg.	Neg.	Neg.	+++	+++	+++	0·64	0·66	0·61
							0·64	0·66	0·61
14	Neg.	Neg.	Neg.	+++	+++	+++	0·64	0·68	0·65
							0·64	0·70	0·66

B. subtilis I 280 mg. dry wt. per exp.
B. subtilis II 480 ,,
B. mesentericus 410 ,,

− No reduction.
+ Slight reduction.
++ Fair reduction.
+++ Pronounced reduction.

Table II. *Hydrolysis of glycogen by bacterial endospores.*

Time in days	Iodine test			Reducing sugar test			Aldose formed (ml. N/100 I)		
	B. subtilis I	*B. subtilis* II	*B. mesentericus*	*B. subtilis* I	*B. subtilis* II	*B. mesentericus*	*B. subtilis* I	*B. subtilis* II	*B. mesentericus*
0	Red-brown	Red-brown	Red-brown	−	−	−	0·28	0·30	0·30
							0·29	0·30	0·30
2	Neg.	Almost neg.	Almost neg.	+	−	−	—	—	—
3	Neg.	Neg.	Neg.	+	+	−	—	—	—
5	,,	,,	,,	++	+++	+	0·48	0·46	0·35
							0·50	0·48	0·35
9	,,	,,	,,	+++	+++	+	0·64	0·48	0·36
							0·64	0·46	0·36
14	,,	,,	,,	+++	+++	+++	0·64	0·48	0·48
							0·64	0·48	0·48

B. subtilis I 280 mg. dry wt. per exp.
B. subtilis II 480 ,,
B. mesentericus 410 ,,

− No reduction.
+ Slight reduction.
++ Fair reduction.
+++ Pronounced reduction.

After the conclusion of the above experiment 25 ml. of the remaining liquid in each flask were filtered through kieselguhr, the clear filtrate was evaporated at reduced pressure to about 3 ml. and the osazone prepared following the directions given by Cole [1933]. In each case typical maltosazone crystals appeared on cooling the liquid. Attempts to hydrolyse 0·2 % maltose solutions with endospore suspensions under toluene were unsuccessful; no increase in the aldose titre was obtained after 2 weeks at 37°.

The proteolytic activity of endospores.

The ability of bacterial endospores to hydrolyse various proteins was next investigated. 2 % solutions or suspensions of the proteins studied were prepared. 10 ml. of such a solution were placed in a 50 ml. Erlenmeyer flask together with

5 ml. of $M/5$ phosphate buffer p_H 7·3, 5 ml. of spore suspension and 2 ml. of toluene. The flasks were corked and incubated at 37°, shaking thoroughly each day, and 1 ml. samples were removed at intervals for formaldehyde titration by the method of Brown [1923]. The results of this experiment which are given in Table III show that bacterial endospores hydrolyse caseinogen, gelatin, egg-albumin, blood-albumin, peptone (Witte), and, in the case of one species, edestin, the number of free amino-groups in the solution increasing in each case. No increase in the initial formaldehyde titration value was obtained when endospores or proteins alone were incubated under similar conditions.

Table III. *Hydrolysis of proteins by bacterial endospores.*

No. of ml. of $N/20$ NaOH required for 1 ml. of the following protein solutions using the formaldehyde titration method of Brown.

Time in days	Caseinogen		Egg-albumin		Blood-albumin		Edestin		Gelatin		Peptone (Witte)	
	B. subtilis I	*B. mesentericus*	*B. subtilis* I	*B. mesentericus*	*B. subtilis* I	*B. mesentericus*	*B. subtilis* I	*B. mesentericus*	*B. subtilis* I	*B. mesentericus*	*B. subtilis* I	*B. mesentericus*
0	0·08	0·08	0·08	0·08	0·08	0·08	0·08	0·08	0·08	0·08	0·18	0·18
7	0·14	0·10	0·08	0·08	0·12	0·09	0·07	0·05	0·10	0·06	0·22	0·20
14	0·19	0·12	0·12	0·10	0·21	0·10	0·08	0·06	0·16	0·14	0·34	0·26
21	0·18	0·14	0·11	0·13	0·20	0·10	0·12	0·05	0·20	0·14	0·33	0·34

The results given are the averages of duplicate experiments agreeing within 5 %.

B. subtilis I 66 mg. dry weight per exp.
B. mesentericus 42 ,,

Summary.

Endospores of aerobic bacilli hydrolyse starch and glycogen to maltose, and various proteins with an increase in the number of free amino-groups in the solution.

REFERENCES.

Brown (1923). *J. Bact.* **8**, 245.
Cole (1933). Practical physiological chemistry. (Heffer, Cambridge.)
Cook (1931). *Zentr. Bakt. Par.* Abt. I (Orig.), **122**, 329.
Ruehle (1923). *J. Bact.* **8**, 487.
Tarr (1932). *J. Hygiene*, **32**, 535.
—— (1933). *Biochem. J.* **27**, 136.
Willstätter and Schüdel (1918). *Ber. deutsch. chem. Ges.* **51**, 780.

LIV. FAT METABOLISM IN FISHES.
III. SELECTIVE FORMATION OF FAT DEPOSITS.

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In a previous communication [Lovern, 1932, 2] data were given of the fatty acid compositions of the glycerides from three depôts in a single fish (*Acipenser sturio*), and some evidence was adduced that a reversible change from palmitic into palmitoleic acid was one factor controlling depôt fat composition. It was also suggested that for the sturgeon the fat in all depôts required a constant ratio of saturated to unsaturated acids. Three other species have now been studied, the conger eel (*Conger vulgaris*), a dolphin (species unfortunately not determined with certainty), and a porpoise (*Phocaena communis*). In the case of the conger eel, the main fat depôt consists of two strips of adipose tissue attached to the peritoneum, whilst the liver is a subsidiary depôt. In order to obtain sufficient liver fat for a full analysis, twelve large fish were used, the peritoneal fats and liver fats being combined. In the case of the dolphin (an immature specimen) and the porpoise (an adult female with a well-developed foetus) the main fat depôt is the blubber. This was divided into two classes in each case; (*a*) that from the body, and (*b*) that from the head, which is mainly found in a thick deposit in a hollow of the skull. These two fats had different compositions. In addition, the jawbones of these animals are filled with an oily pulp, which was extracted, giving a fat of different composition from the blubber fats.

In the hope of extending the range of depôts examined, a semi-micro-method of fatty acid analysis was elaborated. As pointed out previously [Lovern, 1932, 2], for an accurate analysis of a fish oil by macro-methods, roughly 200 g. of fat are required. It was desired to carry out such an analysis on quantities down to 10 g. of fat. A small distilling flask was used, with a bulb of 50 ml. capacity and a side-tube let into the bulb as in the Willstätter flask. A slight constriction in the neck supported a spiral of copper wire, to act as a fractionating column. Quantities as small as 1 g. could be fractionated from this, the residues usually being no more than 0·4 to 0·5 g. Fractions were collected in receivers made from shortened test-tubes, and quantities as low as 0·3 g. were frequently collected and the saponification equivalent and iodine value determined. Iodine values on one quarter of the usual quantity of material are easily performed by the use of proportionately smaller quantities of reagents and titration with more dilute thiosulphate. Equivalent determinations on as little as 0·1 g. of material were carried out. The fat was weighed into a shortened test-tube, 2 ml. of $N/2$ alcoholic potash were added and the mixture was boiled for one hour under reflux with exclusion of carbon dioxide. The contents were washed out with 80 ml. of neutral alcohol into a suitable flask and titrated with $N/50$ sulphuric acid. A good end-point at such dilution depends on the choice of a suitable indicator, *e.g.* dibromothymoltetrachlorophthalein [Cornwell and Esselstyn, 1927]. This has a

very small p_H range (8·8 to 8·4) from deep blue to colourless, and after a little practice, satisfactory duplicates could always be obtained. A blank determination was made as usual.

In every case, as large a quantity of fat as possible (up to 200 g.) was used. Wherever possible, fractional distillations were carried out in the usual Willstätter flasks, and where this was impossible, in the small flask. Equivalent and iodine value determinations were also performed on full quantities where available, and when determined on smaller amounts, the procedure was adapted to make use of as much material as was available. Unfortunately, in the micro-method, it was often impossible to refractionate the fractions from the primary distillation, and this is probably the greatest source of error. In certain cases mixtures have to be taken as containing only two components for the composition to be calculated, and it is almost impossible to obtain only such fractions in one distillation, especially perhaps in this simpler distilling flask. Each fraction however will contain mainly two components, and in many cases only two.

By the use of these micro-methods, analyses were also made of the fats from the blubber of the foetus, and the liver, lungs and heart of the porpoise. In each case only the glyceride fraction was used, the accompanying phosphatides being separated with acetone. The analytical procedure was the same as that adopted in previous cases, except as regards the *iso*valeric acid, which was recovered by steam-distillation. Small quantities of lauric acid also always came over at the same time. The steam-volatile acids were dealt with by ether extraction and fractional distillation, as in the method of Hilditch and Jones [1929], for butter-fats.

The blubber and jaw-fats of the dolphin and porpoise all contain small amounts of waxes, but glycerides preponderate to such an extent that the presence of the waxes can be ignored and the mixed fatty acid composition considered as being essentially that of the glyceride fatty acids. In one case only, that of the head blubber of the dolphin, was the higher alcohol mixture from these waxes examined in detail. It was found to consist mainly of cetyl and oleyl alcohols, with appreciable amounts of tetradecanol and octadecanol. The method used in this analysis was that described by Hilditch and Lovern [1929, 2].

The blubber fats from the members of the porpoise and dolphin family are well-known to be peculiar in respect of the presence of *iso*valeric acid. Gill and Tucker [1930] found in the jaw-oil of a species of dolphin (*Tursiops truncatus*) 86·7 % *iso*valeric acid, 8·4 % palmitic acid and 4·9 % oleic acid. They also found 18·7 % of higher alcohols (mainly dodecanol with some tetradecanol). It must be pointed out that their deduction as to the palmitic and oleic acid percentages was based only on an equivalent and iodine value of the mixed non-steam-volatile acids. By analogy with the results of the present analyses, it seems most improbable that only these two acids were present; indeed, one would expect myristic and palmitoleic to be more important constituents than palmitic and oleic, since the percentage of *iso*valeric acid was exceptionally high, and, in the present results, the higher the percentage of *iso*valeric acid, the larger the proportions of myristic and palmitoleic acids.

The analyses are given in Tables I, II and III, Table I giving the particulars of the oils, and Tables II and III the quantitative compositions of the mixed fatty acids, as weight and molecular percentages respectively.

As in previous papers, the average degree of unsaturation of the C_{18}, C_{20} and C_{22} acids is expressed in terms of lack of hydrogen. The C_{14} and C_{16} unsaturated acids were in every case monoethylenic only. The palmitoleic and oleic acids in each fat were the ordinary $\Delta^{9,10}$ acids, as shown by the production of azelaic acid on oxidation with permanganate in acetone solution.

Table I. *Particulars of fats.*

Species	Fat from	Iodine value	Sap. equiv.	Unsaponifiable %	Type of unsaponifiable
Conger vulgaris	Liver	109·4	—	2·5	Mainly cholesterol
,,	Peritoneum	141·7	—	0·7	,,
Dolphin	Body blubber	136·0	263·4	2·2	Mainly higher alcohols
,,	Head blubber	82·33	228·4	7·5	,,
Phocaena communis	Body blubber	88·82	225·7	2·4	,,
,,	Head blubber	64·76	204·7	2·1	,,
,,	Jaw	44·93	196·3	3·6	,,
,,	Foetal body blubber	108·9	267·8	2·4	Largely cholesterol
,,	Heart	121·3	—	9·7	Mainly cholesterol
,,	Lungs	119·5	—	15·0	,,
,,	Liver	175·0	—	32·1	,,

Table II. *Composition of mixed fatty acids (Wt. %).*

		Saturated					Unsaturated				
Species	Depôt	C_5	C_{12}	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Conger vulgaris	Liver	—	—	5·2	19·2	0·4	0·4	18·0	37·5 (−2·0H)	12·4 (−5·6H)	6·9 (−8·1H)
,,	Peritoneum	—	—	1·8	18·8	0·9	—	6·2	40·6 (−2·1H)	18·3 (−5·4H)	13·4 (−8·4H)
Dolphin	Body	3·2	1·0	7·2	8·6	0·8	4·7	25·9	24·1 (−3·3H)	18·6 (−6·5H)	5·9 (−7·6H)
,,	Head	13·9	2·4	12·5	11·6	0·4	2·7	25·4	15·8 (−2·8H)	12·7 (−5·5H)	2·6 (−7·2H)
Phocaena communis	Body	13·6	3·5*	12·1	4·7	—	4·7	27·2	16·7 (−2·8H)	10·5 (−4·8H)	7·0 (−4·9H)
,,	Head	20·8	4·1	15·8	7·5	0·2	4·6	20·8	15·2 (−2·6H)	9·4 (−4·5H)	1·6 (−4·7H)
,,	Jaw	25·3	4·6*	28·3	4·1	—	3·2	20·3	9·3 (−2·6H)	4·9 (−4·9H)	—
,,	Foetus	1·2	—	14·9	0·6	—	12·3	48·1	15·4 (−4·0H)	7·5 (−7·4H)	—
,,	Liver	—	—	—	7·6	5·5	—	6·1	42·5 (−2·8H)	27·3 (−5·4H)	11·0 (−6·5H)
,,	Lungs	—	—	4·6	9·0	1·2	0·1	16·5	27·0 (−2·4H)	31·0 (−3·3H)	10·6 (−5·4H)
,,	Heart	—	—	8·1	8·2	4·4	4·4	16·8	50·4 (−3·6H)	7·6 (−5·4H)	—

* Trace of lauroleic acid present.

Table III. *Composition of mixed fatty acids (Mols. %).*

		Saturated					Unsaturated				
Species	Depôt	C_5	C_{12}	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Conger vulgaris	Liver	—	—	6·2	20·5	0·4	0·5	19·4	36·3	11·0	5·7
,,	Peritoneum	—	—	2·3	20·8	0·9	—	6·9	40·8	16·9	11·4
Dolphin	Body	8·0	1·3	8·1	8·6	0·7	5·3	26·1	21·9	15·5	4·5
,,	Head	29·2	2·6	11·7	9·7	0·3	2·5	21·4	12·0	8·9	1·7
Phocaena communis	Body	28·7	3·8	11·4	3·9	—	4·5	23·0	12·8	7·4	4·5
,,	Head	39·6	4·0	13·4	5·7	0·1	3·9	16·0	10·5	5·9	0·9
,,	Jaw	44·7	4·1	22·4	2·9	—	2·6	14·4	6·0	2·9	—
,,	Foetus	2·9	—	16·2	0·6	—	13·5	47·0	13·7	6·1	—
,,	Liver	—	—	—	8·6	5·6	—	6·9	43·7	25·7	9·5
,,	Lungs	—	—	5·7	10·0	1·2	0·1	18·4	27·2	28·4	9·0
,,	Heart	—	—	9·5	8·6	4·2	5·2	17·7	48·2	6·6	—

Consideration of these figures reveals several points of interest. In the conger eel we have another instance, as in the sturgeon, of an animal with a liver fat no more unsaturated than the depôt fat. But whereas in the sturgeon both fats have almost equal average unsaturations (iodine value), in the conger eel the liver fat has a much lower iodine value and is much harder. This is wholly due to the different proportions of the various acids present in each case. Unlike the sturgeon, the conger eel has no constant proportion of saturated acids. The oils resemble that from the liver of the halibut in the absence of linoleic acid [Lovern, 1932, 1].

The acids from the dolphin and porpoise blubber and jaw depôts resemble those of the oils from the sperm whale [Hilditch and Lovern, 1928], particularly the head oil of this whale, in that in each case a considerable proportion of the acids have molecular weights below those of the acids taken in the food and are hence products of metabolism. Also the proportions of certain acids which are present in the food are much altered in these depôts, *e.g.* the high proportions of myristic and palmitoleic acids. The oils further resemble the sperm oils in being a mixture of waxes and glycerides, although the proportion of waxes is much lower in the present cases. The occurrence of *iso*valeric acid as a fat constituent is apparently peculiar to the Delphinidae, but lauric acid has been found elsewhere in marine animals [Rees, 1933; Hilditch and Lovern, 1928].

The foetal fat of the porpoise had only a trace of *iso*valeric acid, but large quantities of C_{14} and C_{16} acids, whilst the degree of unsaturation of the C_{18} and C_{20} acids was the highest in the whole series, considerably higher even than that of the maternal liver. The liver fat had the highest average unsaturation, as shown by the iodine value, but this is due largely to the proportions of the different groups of acids (contrasted with the heart fat for instance). It is noteworthy that the organ fats have no *iso*valeric and lauric acids and do not contain unusually large proportions of C_{14} and C_{16} acids. In this respect also the porpoise and dolphin resemble the sperm whale, for Tsujimoto and Kimura [1928] reported the liver fat of the latter to be only glycerides, and with no preponderance of lower acids. This difference from the blubber and jaw fats is particularly emphasised when the results are expressed in molecular percentages.

If the *iso*valeric, lauric and much of the myristic acids, which are presumably metabolic products, had been separately built up into glycerides and then admixed with food fat entering the depôts, we should expect most of these acids to be present as fully-saturated glycerides, which would survive oxidation with powdered permanganate in acetone solution. The body fat of the porpoise which was used contained 10·5 % by weight of fully-saturated material. This was for the most part glycerides, although a small amount of higher alcohols was recovered from it. The composition of the mixed acids is given (wt. %) in Table IV. The significance of these results is best seen by comparing the proportions of each acid present as fully-saturated material.

Table IV. *Proportions of saturated acids combined as fully-saturated glycerides.*

	C_6	C_{12}	C_{14}	C_{16}
Composition of acids in fully-saturated portion	26·1	19·7	42·1	12·1
Reduced to 10·5 total	2·7	2·1	4·4	1·3
Present in 100 parts of original fat	13·6	3·5	12·1	4·7
% of original acid appearing as fully-saturated glycerides	19·9	60·0	36·4	27·7

The results for the lauric, myristic and palmitic acids are in full agreement with those for sperm head oil [Hilditch and Lovern, 1929, 1] in that larger proportions of the lower molecular weight acids are combined as fully-saturated material, but the *iso*valeric acid is an outstanding exception. Only 20 % of it is so combined, the remainder being combined in glycerides which have at least one unsaturated acid in the molecule. That 80 % of the *iso*valeric acid is combined as mixed saturated-unsaturated glycerides was proved by eliminating the two alternative explanations of the oxidation results. One of these is that *iso*valeric acid is itself unstable to oxidation. This was disproved by a second oxidation, which gave the same quantity of fully-saturated material, of the same mean equivalent. The second alternative is that the bulk of the *iso*valeric acid is present as mono- or di-glycerides, which would yield acidic products on oxidation. Gill and Tucker [1930] suggested that in the jaw fat of *Tursiops truncatus* some of it was so combined. A sample of the porpoise body fat was acetylated, and the mean equivalent of the product was 220·0 as against 225·7 before acetylation. This fall is easily accounted for by slight rancidity and the presence of traces of free alcohols, cholesterol, *etc.*, and it is evident that no great quantity of mono- or di-glycerides can be present.

Whatever the origin of the *iso*valeric acid (and this may well be the same as that by which small quantities of this acid are normally produced as waste products in many animals), it can hardly be the same as that of the other fatty acids, all of which have a straight chain structure. The members of the Delphinidae differ from all other animals yet investigated in using this *iso*valeric acid as a constituent of their depôt fats, and it is perhaps also the only case of an acid of an odd number of carbon atoms being so used. In this utilisation of the *iso*-valeric acid, it is largely combined as mixed glycerides with ordinary *n*-fatty acids, and the mode of combination is quantitatively quite different from that of the other saturated acids. In the case of the foetus it appears that *iso*valeric acid was not passed through the placenta, and that the portion found in the foetal blubber was produced by metabolic processes in the foetus itself. A small amount of fat was isolated from the placenta and the glyceride and phosphatide fractions separated. No *iso*valeric acid was present, and the glyceride acids had an iodine value of 117·7 and a mean equivalent of 275·5. Hence no large proportions of C_{14} and C_{16} acids could have been present. The acids from the phosphatide fraction had an iodine value of 96·7, too little being available for the determination of the mean equivalent.

A comparison of the fats from the depôts of each animal (excluding for the moment the organ fats, which are not really depôt fats, and the foetal blubber, which really belongs to a separate metabolic system) shows that the differences are progressive with the molecular weight of the component acids. For instance, in *Phocaena communis* (Table III), on passing from body to head and head to jaw, the *iso*valeric, lauric and myristic acids steadily rise, and the C_{18}, C_{20} and C_{22} acids fall. If the compositions are expressed as total groups (*i.e.* all C_{16} acids being grouped together for instance) in molar percentages, this progressive effect is striking (Table V).

In the conger eel, C_{14} and C_{16} acids are less in the peritoneum than in the liver, and C_{18}, C_{20} and C_{22} are higher. For the dolphin and porpoise the C_5, C_{12} and C_{14} acids increase from body to head and head to jaw, whilst the C_{16}, C_{18}, C_{20} and C_{22} acids decrease. That the extent of these increases and decreases is in general progressive with molecular weight is apparent. The figures for the acids from the head and body blubbers of the sperm whale [Hilditch and Lovern, 1928] show precisely the same relationship.

Table V. *Acids arranged in molecular groups.*

Species	Depôt	C_5	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Conger vulgaris	Liver	—	—	6·7	39·9	36·7	11·0	5·7
,,	Peritoneum	—	—	2·3	27·7	41·7	16·9	11·4
Dolphin	Body	8·0	1·3	13·4	34·7	22·6	15·5	4·5
,,	Head	29·2	2·6	14·2	31·1	12·3	8·9	1·7
Phocaena communis	Body	28·7	3·8	15·9	26·9	12·8	7·4	4·5
,,	Head	39·6	4·0	17·3	21·7	10·6	5·9	0·9
,,	Jaw	44·7	4·1	25·0	17·3	6·0	2·9	—

This suggests that one of the mechanisms operating in the case of these animals to control the type of fat laid down in the different depôts rests on a basis of molecular size and might almost be some form of molecular filtration. Presumably the acids enter the depôts as glycerides and not as free fatty acids, but in either case similar reasoning could be applied. We will suppose for example, that in *Phocaena communis* the size of molecule which can readily pass through the membranes of the jaw depôt is less than that for the head, which in turn is less than that for the body. Under such circumstances a selective deposition of certain of the available glycerides would take place exactly in accordance with Table V. In the less permeable jaw depôt for instance, C_{20} acids could only enter if combined as a triglyceride with two molecules of *iso*valeric acid, and C_{22} acids could not enter at all. More combinations of C_{18} acids would find an entry, *e.g.* one C_{18} radical with two C_5 or two C_{12} radicals, or with one C_5 and a C_{12} or C_{14} radical, and so on progressively for the other acids. What becomes of much of the higher molecular weight acids which the porpoise and dolphin take in their food (this applies also to the sperm whale) is not clear. A small amount will find its way into the organ fats in which lower acids do not preponderate, but it seems not unlikely that much of it is broken down into these same lower acids which are present in such large amounts. The somewhat low degree of unsaturation of the porpoise, dolphin and sperm whale C_{20} and C_{22} acids may be of significance in this respect. This progressive difference in the fat from the different depôts is also shown qualitatively by the characteristics of the fats from the same three depôts of another dolphin (*Lagenorhynchus albirostris*).

Table VI. *Fat from* Lagenorhynchus albirostris.

Depôt	Saponification equivalent	Iodine value
Body	253·9	112·2
Head	246·9	99·81
Jaw	226·1	65·15

Sunderland [1932] examined fats from *Phocaena communis*, making a different separation of the depôts. His samples were from the upper and lower jaws respectively, from the skull and from the blubber. The blubber included both the head and body sections. The fats had different saponification and iodine values, again showing a selective deposition.

If we visualise fat deposition taking place selectively on any basis of permeability differences, we should expect to find, *mutatis mutandis*, that the less permeable depôts (as found by analysis of the fatty acids) would be the smaller in size. Low molecular weight components will find equally easy entry into all depôts, but the larger molecules will only readily enter the more permeable depôts. In actual fact, such is the case. In the conger eel, the less permeable liver depôt contains roughly one-eighth as much fat as the peritoneal depôt. In the dolphin and porpoise, the body fat is much greater in quantity than the head

fat, which in turn is much greater than the jaw fat. The writer has no data for the relative quantities of head and body fat in the sperm whale, but it seems most probable that the body fat is the greater in quantity.

The position in the sperm whale is complicated, however, by the presence of so much wax, which is mainly of low molecular weight compared with triglycerides. Whilst this evidence as to relative size of depôts is in harmony with the molecular filtration theory, it must be borne in mind that some depôts may be restricted by anatomical structure, *e.g.* the jaw depôt of the porpoise. There is also the evidence of Channon and El Saby [1932] that in the herring fat does not necessarily enter all the depôts at the same time and may even be transferred from one depôt to another. It may well be that similar conditions obtain in the animals under discussion.

On this theory of molecular filtration, the saturated and unsaturated acids of the same number of carbon atoms would stand equal chances of entering any particular depôt, unless, of course, there is something peculiar in the mode of combination as glycerides. The only glyceride peculiarity so far evident which bears on this point is the tendency for lower saturated acids to concentrate in the fully-saturated fraction of the fat. Since all the saturated acids present are of relatively low molecular weight, this might conceivably result in a greater chance of saturated acids being found in small glyceride molecules than the corresponding unsaturated acids, and this would be more marked for myristic than for palmitic acid (Table IV). Had the glyceride composition been quite heterogeneous, one would have expected to find the same ratios of palmitic to palmitoleic acid and of myristic to myristoleic acid entering a depôt as were present in the supplies reaching that depôt, and hence the same *ratio* in each depôt, irrespective of the total quantities of these acids. The ratios are given in Table VII. In the case of the conger eel only the C_{16} ratio can be included.

Table VII. *Ratio of saturated to unsaturated acid.*

Species	Depôt	C_{14} acids	C_{16} acids
Conger vulgaris	Liver	—	1·1
,,	Peritoneum	—	3·0
Dolphin	Body	1·5	0·33
,,	Head	4·6	0·46
Phocaena communis	Body	2·6	0·17
,,	Head	3·4	0·36
,,	Jaw	8·8	0·20

The differences in the ratios are very large in certain cases, especially for the conger eel and for C_{14} acids in the dolphin and porpoise. With one exception the differences for the dolphin and porpoise are all in the same direction, an increasing ratio of saturated to unsaturated acid on passing from body to head and head to jaw. This is in agreement with the glyceride peculiarity. The jaw of the porpoise is an exception for the C_{16} acids, and in the conger eel there is a big difference in the opposite direction, *i.e.* a higher ratio in the more permeable depôt, which cannot be explained by glyceride structure.

The interpretation which seems most in accordance with all the facts is that the saturated and unsaturated acids of the same number of carbon atoms are interconvertible, as suggested in the previous paper for the sturgeon [Lovern, 1932, 2]. It would follow that the conversion takes place in the depôt itself. Support of the idea of interconvertibility is found in the work of Mazza and Stolfi [1933] and of Berend [1933] who have described dehydrogenases which act on higher fatty acids.

The peculiar composition of the porpoise and dolphin depôt fats raises some interesting questions. Where, for instance, is the site of the production of the lower fatty acids, due perhaps to breakdown of the C_{20} and C_{22} acids? Also of the *iso*valeric acid, which is apparently produced quite separately, and combined into mixed glycerides in a different manner from the other lower acids? That this site is the liver seems most unlikely, in view of the apparent entire absence of these acids from it—indeed, the liver fat closely resembles the other organ fats examined. In fact, the organ and the depôt fats are so thoroughly different in their whole composition that it is difficult to imagine the liver playing any large part in the metabolism of these depôt fats. An organ that is worthy of attention in this respect is the spleen, in view of its function of removing fat from the blood during alimentary lipaemia [Marino, 1933], and hence its rôle as an intermediary in the formation of depôt fats from food fat [*cf.* also Leites *et al.*, 1933].

In the case of the foetus it seems probable that the fat received from the maternal system is mainly of the ordinary marine type. This is modified by the foetus in two ways—by production of much C_{14} and C_{16} acids in place of C_{20} and C_{22}, and by admixture of *iso*valeric acid. The metabolic process from which the *iso*valeric acid arises evidently does not function to any great extent up to this stage. The characteristic composition of the foetal fat is further evidence that the *iso*valeric acid and the lower *n*-acids are produced by separate processes.

The molecular selectivity displayed in the formation of these depôt fats does not appear to hold for all species, *e.g.* the pike and the sturgeon [Lovern, 1932, 1, 2]. In these animals the different depôts appear equally permeable, resulting in the constant proportion of total C_{16} acids found for the sturgeon and the pike.

Summary.

The liver and peritoneal fats of the conger eel, and the fats from a number of depôts and organs of a dolphin and a porpoise have been examined. Considerable differences in fatty acid composition are found in the different fats from one animal, indicating a highly selective formation of the fat deposits.

The porpoise and dolphin fats show great similarity of type to those from the sperm whale, thus affording further evidence that zoologically related species exhibit definite similarities in fat composition.

The porpoise and dolphin depôt fats are unique in containing large amounts of *iso*valeric acid and the mode of combination of this is peculiar.

The depôt fats of the porpoise and dolphin are quite different in type from the organ fats of the same animals. Whilst no explanation of this can be offered, it is not in harmony with the idea of fat transfer to the liver before utilisation. The theory of desaturation in the liver is also unsupported by the conger eel and porpoise figures. In the case of the porpoise, the heart fat contains an even more unsaturated group than the liver fat.

The blubber of a porpoise foetus showed a peculiar composition and a very high degree of unsaturation.

The depôt fats of all the animals show a molecular size gradation which suggests that one cause of specificity may be a molecular filtration.

Some further evidence is adduced in support of the idea of interconvertibility of saturated and unsaturated acids of the same number of carbon atoms.

REFERENCES.

Berend (1933). *Biochem. Z.* **260**, 490.
Channon and El Saby (1932). *Biochem. J.* **26**, 2021.
Cornwell and Esselstyn (1927). *J. Amer. Chem. Soc.* **49**, 826.
Gill and Tucker (1930). *Oil and Fat Ind.* **7**, 101.
Hilditch and Jones (1929). *Analyst*, **54**, 75.
—— and Lovern (1928). *J. Soc. Chem. Ind.* **47**, 105 T.
—— —— (1929, 1). *J. Soc. Chem. Ind.* **48**, 359 T.
—— —— (1929, 2). *J. Soc. Chem. Ind.* **48**, 365 T.
Leites, Koslowa and Jussin (1933). *Deutsch. med. Woch.* **59**, 214.
Lovern (1932, 1). *Biochem. J.* **26**, 1978.
—— (1932, 2). *Biochem. J.* **26**, 1985.
Marino (1933). *Arch. Farmacol. Sper.* **55**, 289, 327.
Mazza and Stolfi (1933). *Atti R. Accad. Lincei*, **17**, 476.
Rees (1933). *Analyst*, **58**, 222.
Sunderland (1932). *Biological Board of Canada. Progress Reps. Pacific Biol. Stn. and Fisheries Exp. Stn.* No. 14, 14.
Tsujimoto and Kimura (1928). *Chem. Umschau*, **35**, 317.

LV. A NOTE ON THE STRUCTURE AND CHEMISTRY OF GLUCURONIC ACID MONOBENZOATE.

By ARMAND JAMES QUICK.

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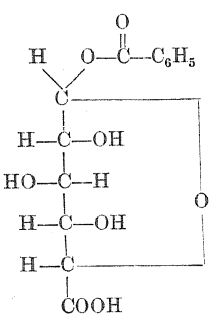
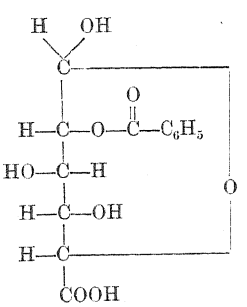
(*Received January 25th, 1934.*)

ALTHOUGH glucuronic acid was discovered over 50 years ago, its position in physiology remained until recently undefined. Now, however, it seems fairly certain that this compound is intimately related to carbohydrate metabolism. The depancreatised dog can still produce glucuronic acid from the glycogenetic fraction of the protein molecule, which would otherwise go to glucose and be excreted [Quick, 1926, 2]; furthermore, insulin can markedly increase while acetoacetic acid as well as lactic acid decidedly suppress its output [Quick, 1932]. The importance of glucuronic acid depends not only on its relationship to carbohydrates, but also on the fact that it represents a type of conjugation, which is commonly referred to as a detoxication mechanism. In the opinion of the writer these synthetic processes are constantly active in normal metabolism, but they often only become manifest when the organism applies them to abnormal substances. It is probable that the uric acid-carbohydrate compound normally present in the blood is produced by a conjugating mechanism and may represent an important step in the excretion of uric acid.

The desirability of studying the molecular structure and chemistry of the various conjugated glucuronic acids is evident. Recently Pryde and Williams [1933] reported studies on the structure of bornyl-*d*-glucuronide, glucurone, and the glucuronic acid-benzoic acid compound. This last compound was prepared and studied by Quick [1926, 1]. From the data then obtained, he concluded that the compound was not benzoylglucuronic acid as Magnus-Levy [1907] had postulated (I) but glucuronic acid monobenzoate (II).



I



II

Pryde and Williams, however, question the ester structure and believe that the formula originally proposed by Magnus-Levy is the correct one. The writer's contention that the compound has formula II is based in part on the finding that sodium cyanide reacts directly with glucuronic acid monobenzoate, thus indicating that the aldehyde group is free and is not combined with benzoic acid. Although Pryde and Williams obtained the same polariscopic changes as the author, they claim that the alkalinity of the sodium cyanide first caused a hydrolysis, and that it was the liberated glucuronic acid which reacted. They base their conclusions on the qualitative detection of benzoic acid, which obviously can be misleading, and was unfortunate in view of the fact that satisfactory quantitative methods were available, which would have prevented a hasty and erroneous conclusion.

In order to redemonstrate that sodium cyanide reacts with glucuronic acid monobenzoate directly without previous hydrolysis, the following experiment was performed.

0·4 g. of glucuronic acid monobenzoate was dissolved in 20 ml. of water and mixed with a solution containing 0·09 g. sodium cyanide in 20 ml. The resulting mixture was faintly alkaline to phenolphthalein and became progressively more so as the reaction progressed. At the end of 21 hours, the solution was acidified with hydrochloric acid until acid to Congo red, then extracted with 4 portions (25, 25, 15, 10 ml.) of neutral toluene and the benzoic acid determined in the usual manner by titration with 0·1 *N* sodium ethoxide. The amount of free benzoic acid found was 0·0145 g. while the total amount contained in 0·4 g. of glucuronic acid monobenzoate is 0·1635 g. Therefore only 8·9 % of the compound was hydrolysed in 21 hours. At the same time, a determination of the reducing action made on an aliquot by the method of Shaffer and Hartmann [1920–21] showed that only 3·9 % of the original reducing power remained, indicating that 96·1 % of the aldehyde had reacted to form the cyanohydrin derivative. It must be concluded that the benzoic acid is not attached to the aldehyde group, for, if it were, an amount of benzoic acid equivalent to the quantity of cyanohydrin produced should be formed. Therefore the benzoic acid must be linked to one of the hydroxy-groups of glucuronic acid.

Although the experiment presented answers the criticism of Pryde and Williams there are sufficient data in the writer's original paper to support adequately the claim that the compound under discussion is not benzoylglucuronic acid but a glucuronic acid monobenzoate.

In the first place, when 0·2 g. of glucuronic acid monobenzoate (which requires 0·033 g. of sodium cyanide) was treated with a slight excess, *i.e.* 0·045 g. of sodium cyanide, the solution was alkaline to phenolphthalein and became more alkaline as the reaction progressed. If hydrolysis had occurred, the solution should have become acid, since the amount of benzoic acid liberated would have greatly exceeded the amount of base available for neutralisation.

In the second place, glucuronic acid monobenzoate showed mutarotation in an alkaline solution. As the writer expressly stated, this change of rotation occurred with scarcely any hydrolysis.

Further study is required to determine by which hydroxy-group the benzoic acid is linked to glucuronic acid. Probably the most promising approach to the problem is the preparation of the methyl ester methylglucoside, as described by the writer, before attempting complete methylation.

SUMMARY.

The compound resulting from the conjugation of glucuronic acid with benzoic acid is a glucuronic acid monobenzoate, and not benzoylglucuronic acid, for it acts directly with sodium cyanide to form the cyanohydrin, and it exhibits mutarotation.

REFERENCES.

Magnus-Levy (1907). *Biochem. Z.* **6**, 502.
Pryde and Williams (1933). *Biochem. J.* **27**, 1197, 1205, 1210.
Quick (1926, 1). *J. Biol. Chem.* **70**, 59.
—— (1926, 2). *J. Biol. Chem.* **69**, 549.
—— (1932). *J. Biol. Chem.* **98**, 537.
Shaffer and Hartmann (1920–21). *J. Biol. Chem.* **45**, 365.

LVI. THE RELATION BETWEEN THE ACTIVITY OF UREASE AND THE OXIDATION-REDUCTION POTENTIAL.

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(*Received January 30th, 1934.*)

Amongst the substances which exercise accelerating or inhibitory actions on the activity of enzymes, particular interest has recently been attached to compounds possessing reducing or oxidising properties such as hydrogen sulphide, cysteine, glutathione, iron and other metallic complexes, and more recently also ascorbic acid [Karrer and Zehender, 1933; Edlbacher *et al.*, 1933, 1, 2]. These substances may react chemically with the enzyme-substrate system or they may influence the enzyme reaction by stabilising the oxidation-reduction potential of the medium. In the first case the activity will be related to the particular properties of the system added, whilst in the latter case every oxidation-reduction system will maintain the same potential and have the same influence. Mainly Waldschmidt-Leitz *et al.* [1929; 1930; 1933] and Grassmann *et al.* [1930; 1930–31] have indicated a relationship between the oxidation-reduction potential and the activity of the enzyme. Obviously a method of elucidating this problem would be to investigate the activity of the enzyme in the presence of different oxidation-reduction systems which maintain the potential (r_H) at known levels but which do not react chemically in any other way with the constituents of the mixture—in other words to impose conditions similar in character to those employed to maintain a desired hydrogen ion concentration in a reaction mixture.

There is however a difficulty in that a very large number of different oxidation-reduction systems are necessary in order to cover the entire range of the r_H scale. Each of the common oxidation-reduction systems is suitable only for a range of about 4 r_H units, whereas the full range between the hydrogen and oxygen electrodes is about 40 units. For this reason approximately ten systems are necessary in order to obtain a complete curve covering relation between activity and r_H. However, the task can be restricted considerably by making a series of studies spaced over the whole range.

As a first example the activity of urease was investigated. The reason for this was that Quastel [1933] has recently found a number of substances belonging to the class of polyphenols (quinol, catechol, pyrogallol, adrenaline, *etc.*) all of which exerted a strong inhibitory action on urease. The corresponding quinones were found to be more toxic than the phenols. The inhibition could be counteracted by hydrogen sulphide and other thiol compounds. Quastel was of opinion that only the quinone actually inhibited the enzyme, and that such inhibition as was exhibited by the phenol was actually due to the presence of traces of quinone.

Since however these substances all form fairly strongly positive reversible oxidation-reduction systems and since other oxidising agents such as hydrogen peroxide are actually inhibitors it seems reasonable to suspect a relationship between the activity of the enzyme and the oxidation-reduction potential of the system.

In the present investigation the hydrolysis of urea by urease was studied in the presence of different types of reversible oxidation-reduction systems. In particular, it was important to study that part of the oxidation-reduction scale which is poised by the system quinol-quinone, that is to say the positive section. In addition a study was made of conditions at the negative end of the oxidation-reduction scale. The experimental difficulty of carrying out investigations with the very oxygen-sensitive negative systems, for example rosindulin or neutral red, is very great. Therefore the action of the enzyme was studied in a stream of hydrogen in the presence of an active catalyst in the form of palladised asbestos. Also, in the studies of the readily autoxidisable leucomethylene blue a stream of hydrogen was maintained through the apparatus while the hydrolysis was in progress. Other experiments were carried out in which a stream of air was employed and in which the reaction mixture was kept in a sealed vessel. The degree of hydrolysis of the urea was measured either by estimating the ammonia by aeration in the usual manner or by direct titration as follows: to a sample of the test solution an excess of standard trichloroacetic acid was added; the mixture was made up to a certain volume and filtered, and an aliquot part of the filtrate was titrated with $N/10$ NaOH to p_H 4·4 methyl orange being used as indicator. At this point the phosphoric acid used as a buffer had its first neutralisation point and consequently a minimum of buffering power. Free trichloroacetic acid being a strong acid can be titrated with this indicator without difficulty whereas ammonium salts do not give the acid colour.

The hydrogen employed was prepared in a Kipp's apparatus and purified by washing with potassium permanganate, mercuric chloride and alkaline pyrogallol. Air was purified by washing with acid in order to remove traces of ammonia. In all experiments in which a stream of gas was employed the outlet tube of the apparatus led into a vessel containing standard acid which was titrated for the estimation of the ammonia liberated. The enzyme preparation employed was either freshly ground soya bean or a 10 % aqueous extract thereof. The urea was purified by crystallisation. The palladised asbestos was prepared by carefully treating a warm suspension of finely divided asbestos in a very dilute solution of palladium chloride with glucose and sodium hydroxide.

Influence of air and hydrogen.

It was necessary to investigate whether any alteration of activity of urease could be detected in experiments in which air or hydrogen was bubbled through the solution, since Edlbacher *et al.* [1932; 1933, 1, 2] has detected a difference in the case of arginase. Similar reaction vessels containing 10 ml. of 6 % urea solution; 5 ml. M phosphate buffer p_H 7·4; 10 ml. 10 % aqueous soya bean extract; and 25 ml. distilled water, were set up. One was aerated at room temperature with air, whilst through another a stream of hydrogen was passed for the same time. In both cases the gas streams were regulated so that approximately one bubble per second passed through the solution, this being done in order to prevent frothing which might induce inactivation of the enzyme.

The following table gives the values of ammonia formed by fermentation in various experiments with different enzyme preparations (in ml. $N/10$ NH_4OH).

Exp. no.	Time min.	Closed vessel	Air stream	Hydrogen stream
1	85	4·65, 4·80	4·50, 4·65	—
2	205	9·35, 9·50	9·15, 9·05	—
3	95	4·30	—	4·40
4	220	9·15	—	9·05
5	95	—	9·80	10·00
6	40	—	5·20	5·40

This shows no significant difference between the tests.

Influence of active hydrogen.

In this experiment the reaction vessel contained 0·05 g. of palladised asbestos suspended with the enzyme preparation in the buffered medium. Hydrogen was passed through the mixture for 15 minutes after which the urea was added. In this manner it was ensured that the potential of the reaction mixture would be adjusted to the negative level of the hydrogen electrode at the p_H employed. At a temperature of 18° this is −427 mv. (referred to normal hydrogen electrode). The ammonia production was compared with that of two controls in closed vessels, one of which contained the same amount of catalyst.

The results of several experiments are:

Exp. no.	Time (min.)	Catalyst In hydrogen stream	Catalyst Closed vessel	Closed vessel
		Titrations in ml. $N/10$ NH_4OH		
7	85	—	7·45	7·75
8	205	—	13·70	15·30
9	85	—	7·05	7·45
10	205	—	13·95	14·70
1	85	4·45, 4·40	—	4·65, 4·80
2	205	8·85, 8·60	—	9·35, 9·50

This indicates a slight inhibition of the enzyme in the presence of the catalyst employed.

Influence of methylene blue.

Quastel [1932] has shown that methylene blue does not affect unpurified preparations of urease but that it exerts a strongly inhibiting action on the purified enzyme. Reaction vessels were set up, each containing 2 ml. 6 % urea solution, 30 ml. $M/5$ phosphate buffer p_H 7·4, 10 ml. 10 % soya bean meal extract. To one were added 10 ml. of 0·15 % methylene blue in $M/50$ phosphate buffer, to another the same solution reduced by hydrogen in the presence of palladised asbestos. The former experiment was aerated, whilst in the latter case a stream of hydrogen was employed. To a control 10 ml. of the buffer only were added. Hydrolysis was allowed to occur at room temperature.

Results of the ammonia estimations with different enzyme preparations in various times are given in the following table:

Exp. no.	Time min.	Methylene blue oxidised	Methylene blue reduced	Control
		Titrations in ml. $N/10$ NH_4OH		
11	90	17·2	16·9	—
12	80	4·55, 4·60	—	4·70, 4·60
13	120	7·30	—	7·30
14	60	13·70	—	13·10

If it be assumed that approximately 10 % of the methylene blue in the reduced preparation was present in the oxidised form the potential of this preparation can be regarded as −28 mv. (referred to the normal hydrogen electrode); that of the oxidised preparation (99 % oxidised) may be taken as

+58 mv. (The normal potential of the system methylene blue-leucomethylene blue at p_H 7·4 is zero.) It may be concluded, therefore, that the activity of the urease preparation employed is unaffected by potentials within the range −28 to +58 mv.

Ferro-ferricyanide system.

This system possesses a normal potential of approximately +450 mv. which is unaffected by hydrogen ion concentration. In the first place both extremes of the potential range were investigated. The potentials are not very sensitive to oxygen so that they can be measured electrometrically in the presence of air. As the substances are quite stable the hydrolyses were carried out in closed vessels kept in an incubator at 37°. Each vessel contained in a total vol. of 50 ml. 10 ml. 6 % urea solution; 5 ml. *M* phosphate buffer p_H 7·4; 5 ml. 10 % soya bean meal extract and 1 millimol of either ferro- or ferricyanide. Controls without addition were set up.

The respective ammonia productions in various experiments were:

Exp. no.	Time min.	Ferro-cyanide	Ferri-cyanide	Controls without addition
		Titrations in ml. *N*/10 NH_4OH		
7	85	7·70	7·15	7·75
8	205	15·20	13·90	15·30
9	85	7·45	6·80	7·45
10	205	14·75	13·65	14·70

The oxidation-reduction potentials were measured with gilded platinum electrodes against a standard calomel electrode. Owing to inadequate poising the readings were not quite steady; the values for ferrocyanide ranging between −22 and +10 mv., those for ferricyanide between +264 and +336 mv. Referred to the normal hydrogen electrode these values become +228 to +260 mv. (ferrocyanide) and +514 to +586 mv. (ferricyanide). The rate of hydrolysis of urea was unaffected by these substances, apart from a very slight inhibition exerted by the ferricyanide.

Influence of quinol.

The poisoning of urease by quinol which was observed by Quastel, and which can be demonstrated both with the crude soya bean meal and with extracts, can, as he also showed, be prevented by the addition of substances containing the SH group. The inhibition thus removed could be prevented likewise by treatment with active hydrogen. Three experiments were set up. In each the reaction vessel contained in a total volume of 50 ml. 10 ml. 6 % urea solution; 5 ml. *M* phosphate buffer solution p_H 7·4; 10 ml. of a 10 % aqueous soya bean meal extract or the corresponding quantity of soya bean meal. To the contents of two vessels 0·1 g. quinol was added, the third serving as a control. One of the vessels containing the quinol was exposed to a stream of hydrogen in contact with 0·05 g. palladised asbestos for 15 minutes before the enzyme preparation was added, and for a further 15 minutes afterwards. The urea solution was then added and the stream of hydrogen maintained during the hydrolysis.

Results of ammonia estimations in various experiments are shown in the following table:

Exp. no.	Time min.	No. 1 quinol Pd-catalyst in hydrogen stream	No. 2 quinol (closed vessel)	No. 3 control
		Titrations in ml. *N*/10 NH_4OH		
15	125	4·30	0	4·55 } soya bean extract
16	210	8·80	0	9·15 } soya bean extract
17	120	6·50	0·90	7·05 meal

The oxidation-reduction potential in No. 2 was between −53 and −34 mv. measured against the standard calomel electrode (*i.e.* +197 to +216 mv., referred to the normal hydrogen electrode).

It is apparent therefore that there is almost complete inhibition of urease in the presence of quinol and that this is prevented by reduction with hydrogen in the presence of catalytic palladium.

Discussion.

The activity of urease was found to be unaffected by the oxidation-reduction potential of the medium within the range investigated—namely, that covered by the systems palladium-hydrogen, methylene blue-leucomethylene blue, and ferri-ferrocyanide. Inhibition by quinol is apparently a specific action which is not related to the oxidation-reduction potential. It can probably be ascribed, as Quastel has suggested, to the action of traces of quinone rather than to the quinol itself, because no inhibition occurs when a stream of hydrogen is passed in the presence of palladium. It can be concluded that the reaction of the enzyme with quinone, if it is oxidative in nature, is not reversible in the thermodynamic sense; otherwise ferricyanide with a potential of more than +500 mv. would exert a stronger action than that of quinol with only +200 mv.

Summary.

1. A stream of hydrogen or air did not affect the activity of urease. Moreover, activation of the hydrogen by palladium-asbestos had no appreciable influence on the rate of hydrolysis of urea.

2. The reversible oxidation-reduction systems, methylene blue and ferro-ferricyanide, did not influence the activity of the enzyme.

3. Quinol inhibits urease action, but on reduction with active hydrogen this effect disappears. It is believed that the original inhibition is due to a chemical reaction with the enzyme rather than to an influence on the oxidation-reduction potential.

The author wishes to express his thanks to Prof. J. B. S. Haldane and Prof. J. C. Drummond for giving the facilities of carrying out this work and for much valuable criticism and advice.

REFERENCES.

Edlbacher, Kraus and Walker (1932). *Z. physiol. Chem.* **206**, 65.
—— —— and Leuthardt (1933, 1). *Z. physiol. Chem.* **217**, 89.
—— —— and Leuthardt (1933, 2). *Klin. Woch.* 1843.
Grassmann, Dyckerhoff and Schoenebeck (1930). *Z. physiol. Chem.* **186**, 183.
—— Schoenebeck and Eibeler (1930–31). *Z. physiol. Chem.* **194**, 124.
Karrer and Zehender (1933). *Helv. Chim. Acta*, **14**, 701.
Waldschmidt-Leitz, Bek and Kahn (1929). *Naturwiss.* **17**, 85.
—— and Schäffner (1930). *Naturwiss.* **18**, 280.
—— Scharikova and Schäffner (1933). *Z. physiol. Chem.* **214**, 75.
Quastel (1932). *Biochem. J.* **26**, 1685.
—— (1933). *Biochem. J.* **27**, 1116.

LVII. DEHYDROGENASES OF THE FILTRABLE ORGANISM OF *AGALACTIA*.

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(*Received February 15th, 1934.*)

THE detection of dehydrogenases in *Agalactia* has been reported in a previous paper [Pirie and Holmes, 1933] where the great instability of the enzymes has also been demonstrated. The experiments reported in this paper were undertaken in order to see how many substrates the organism could oxidise. *Agalactia* and *Pleuropneumonia* are the smallest organisms so far shown to possess enzymes, and it seemed possible that the number of enzymes might be very restricted and their kinetics rather different from those of ordinary bacterial enzymes. For the sake of simplicity *Agalactia* will be described as a virus in this paper in spite of the paper by Ledingham [1933] in which many different forms of the organism are described. This pleomorphism does not entirely differentiate *Agalactia* from viruses since Bedson [1933] has described a life cycle for the virus of psittacosis which involves many different and some large forms. While admitting that the exact classification of *Agalactia* is still in doubt the fact of its filtrability enables it to be considered as a very small organism.

The experimental procedure has been described fully in the previous paper. *Agalactia* has been grown on Hartley's broth *plus* 5–10 % horse-serum at 37° for 3 days. In order to obtain the virus free from the medium the culture was centrifuged at 3500 r.p.m. for one hour and the sediment was drained and rubbed up in saline. The volume of saline was usually 1/10th that of the original culture. The enzymes of this suspension could then be investigated. The oxygen uptake and the methylene blue reduction times in the presence of different substrates were measured. In every experiment a saline control and also a control of the virus *plus* some substrate which was known already to be oxidised were included since occasionally apparently well-grown cultures failed to show any dehydrogenases whatever. Table I shows the results obtained using the methylene blue technique. These indicate that a considerable number of substrates are oxidised by *Agalactia* if the substrate concentration is high enough. *Agalactia* oxidises very few compounds if their final concentration is less than $M/10$. Lactate, adenylic acid, adenosine, fructose and hexosediphosphate are the only substrates which are oxidised in low concentration. In this respect *Agalactia* resembles *Esch. coli* since Quastel and Whetham [1925] found that the dehydrogenases of the resting bacterium varied enormously in their affinity for their substrate and relatively few enzymes attacked substrates in low concentrations. The significance of enzymes which do not attack a substrate unless it is in high concentration is obscure. It seems possible that it is a question of incomplete specificity of enzymes so that each dehydrogenase has more than one substrate but has enormously varying affinities for the different substrates which it may attack. Table II shows the effect of variation of substrate concentration on the velocity of reduction of methylene blue.

Table I. *Dehydrogenases of* Agalactia.

Each Thunberg tube contained 1·0 ml. virus suspension, 1·0 ml. substrate, 1·0 ml. *M*/10 phosphate buffer of p_H 7·4 and 0·2 ml. 1/3000 methylene blue. The final substrate concentrations are given.

Substrate	Red. time in mins.	Substrate	Red. time in mins.
Broth	11, 6	Formate *M*/6	60
Serum	10	Succinate *M*/6	39
Lactate *M*/20	7, 11	Malate *M*/6	12
Glucose *M*/6	31	Oxalate *M*/6	∞
Fructose *M*/6	14	Gluconate *M*/6	∞
Galactose *M*/6	18	Glutamate *M*/12	56
Lactose *M*/6	24	Aspartate *M*/12	40
Adenylic acid *M*/300	17	Alanine *M*/6	48
Adenosine *M*/300	24	Citrate *M*/6	42
Adenine (saturated)	∞	Hexosediphosphate *M*/300	25
Xanthine	∞	Hexosemonophosphate	∞
Saline	∞		

Table II. *Effect of variation of substrate concentration on the reduction time of methylene blue.*

Conditions the same as in Table I.

Substrate	Conc.	Red. time	Substrate	Conc.	Red. time
Glucose	1/3 saturated	30	Lactose	*M*/6	38
	M/6	32		*M*/30	∞
	M/30	∞	Galactose	*M*/6	39
Fructose	*M*/3	14		*M*/30	60
	M/30	28		*M*/100	∞
	M/300	23	Succinate	*M*/6	46
Lactate	*M*/30	24		*M*/30	∞
	M/300	20	Aspartate	*M*/12	43
	M/1500	85		*M*/60	120

Alternatively it suggests that a co-enzyme is necessary for the reaction or that an intermediate compound, not the actual substrate added, is the true substrate for the enzyme. The effect of the addition of the co-enzyme for liver glucose dehydrogenase, described by Mann [1932], on the velocity of glucose oxidation by *Agalactia* was investigated. It was found that the co-enzyme preparation itself was rapidly oxidised and that no acceleration of glucose oxidation could be shown. The reduction time of a mixture of glucose and co-enzyme was approximately the mean of the reduction times for the separate substrates as though only one enzyme were concerned. The same is true for mixtures of fructose or galactose with the co-enzyme or with adenylic acid. Since *Agalactia* dehydrogenates adenosine but has no effect on adenine it is presumably the carbohydrate part of the molecule that is attacked. There seems to be only one enzyme in *Agalactia* which oxidises all the different carbohydrates since a mixture of any two of these is oxidised at the mean rate of oxidation of the two separately. It is possible that the co-enzyme of glucose dehydrogenase also contains a carbohydrate radical since it is oxidised by the same enzyme (Table II).

The addition of phosphate buffer to the mixture of virus and substrate in the vacuum tube often greatly decreased the reduction time. This was at first thought to be a specific effect of phosphate since borate and sulphate had no accelerating effect. Quastel and Whetham [1924] state that replacement of phosphate by borate had no effect on the activity of succinic dehydrogenase of *Esch. coli*. But it seems more probable that the accelerating action of phosphate

Table III. *Unity of carbohydrate dehydrogenases of* Agalactia.

Final conc. of total sugars *M*/4. Reduction times in minutes.

		Calc. mean			Calc. mean			Calc. mean
Glucose	31	—	Fructose	14	—	Lactose	24	—
+ Fructose	16	22	+ Glucose	16	22	+ Glucose	24	27
+ Lactose	24	27	+ Lactose	16	19	+ Fructose	16	19
+ Galactose	21	24	+ Galactose	14	16	+ Galactose	21	21
Galactose	18	—	Fructose	33	—	Glucose	32	—
+ Fructose	14	16	+ Co-enzyme	28	28	+ Co-enzyme	26	27
+ Glucose	21	24	Fructose	43	—	Glucose	32	—
+ Lactose	21	21	+ Adenylic acid	24	32	+ Adenylic acid	25	27

on the enzymes of *Agalactia* is a p_H effect, since some of the dehydrogenases have a sharp optimum p_H of 7·4. In vacuum tubes containing no buffer the p_H rises during evacuation and therefore the enzyme may not be acting under optimum conditions. It was impossible to be certain of this point since experiments with citrate and glycylglycine as buffers were inconclusive. Those enzymes which seemed most affected by phosphate were also the most unstable and were often destroyed after the suspension of *Agalactia* had stood on the bench for half an hour. In order to test the relative effects of different buffers all vacuum tubes had to be evacuated simultaneously. Since it seemed possible that phosphate had either a stabilising or stimulating action on some of the enzymes of *Agalactia* it was thought that it might have a similar action on the organism itself. The sediments from equal volumes of culture were suspended in equal volumes of *M*/10 phosphate buffer of p_H 7·4 or in physiological saline of the same p_H. The suspensions were kept on the bench and the activity of some dehydrogenases was determined at intervals. It was found that the glucose and succinic dehydrogenases were destroyed more rapidly in phosphate than in saline whereas the lactic dehydrogenase was equally stable in either solution. Elford and Galloway [1933] found that the virus of louping-ill was less stable, as judged by its pathogenicity, in phosphate solutions than in saline and it would be tempting to consider that this case also is one of enzyme destruction.

The dehydrogenases of *Agalactia* varied considerably in their relative activities. Many cultures were investigated under standard conditions before this variability was accepted as being a real occurrence not due to chance changes in technique. The ratio of the glucose and lactic dehydrogenases was particularly investigated. First of all the ratio of the two enzymes at different stages of growth of the culture was estimated. 30 ml. samples were removed from cultures on the 2nd, 3rd and 4th days of growth and centrifuged for one hour, and the lactate and glucose dehydrogenase activity was estimated in the usual way. A saline control was also put up, the virus sediment being rubbed up in 3·0 ml. saline. If an active glucose dehydrogenase was present the ratio of the enzymes was usually 2–3 to 1 at all stages of growth, but if a very feeble one only was detectable this seemed to be at its maximum on the 3rd day of growth when the ratio might be 7:1.

The variability depended more noticeably on the medium. Different batches of Hartley's broth gave very different glucose dehydrogenases although the lactic dehydrogenase was constant. In order to see if the medium were the controlling factor in variability of the enzymes a culture grown on a broth which tended to diminish the glucose enzyme was transplanted to a "good"

broth. The enzyme activity increased considerably. After two cultures on this broth a subculture was made on to the "bad" broth and the enzymes were estimated again. There was a tendency for the glucose and other dehydrogenases to disappear in the first subculture, the lactic dehydrogenase being the only one left. After several generations the enzymes might return even on the bad medium. Variability therefore seems to be neither completely a matter of medium nor a matter of phase of growth. It is possible that different media

Table IV. *Variability of enzymes.*

(*a*) Comparison of enzymes on two broths.

	Type of broth		
Substrate	"Bad"	"Good"	"Bad"
Control	∞	101	∞
Glucose *M*/1	∞	48	∞
Lactate *M*/10	14	28	12
Formate *M*/2	182	39	∞
Fructose *M*/10	180	—	—

(*b*) Glucose and lactate dehydrogenases of successive cultures on different broths.

	Type of broth			
Substrate	"Good"	"Bad"	"Bad"	"Bad"
Saline	200	28	101	138
Glucose *M*/1	36	28	100	70
Lactate *M*/10	26	6	8	10

Conditions are the same as in previous tables. Reduction times are given in minutes.

influence the preponderance of one form of the organism over any other [Ledingham, 1933]. Microscopical preparations of the cultures by Ledingham's method for the detection of the different forms was not carried out, but bacterial sterility was tested by inoculation into broth and by Gram staining of the sediment from the centrifuged culture.

A more detailed study of the lactic dehydrogenase of *Agalactia* was made since this enzyme seemed to be the most active and stable one possessed by the virus. Quastel found that the lactic dehydrogenase of *Esch. coli* was inhibited by other acids containing the groups —CHOH.COOH or —CO.COOH. The effect of such acids on the activity of the lactic enzyme of *Agalactia* was investigated in order to see if this was of the same type as the enzyme contained in *Esch. coli*. The solution of the inhibitor was added to the virus before the lactate in order that it might be adsorbed at the surface non-competitively. The time taken by the virus to reduce methylene blue in the presence of lactate, the inhibiting acid and a mixture of the two was then determined. Solutions of oxalate and gluconate inhibited lactic dehydrogenase almost completely while glycollic and glyceric acids had slight inhibitory actions. Glucose, citrate, tartrate and malate had no effect. Gluconate or oxalate was added to the medium in which *Agalactia* was grown in order to see if any depressing action on the growth of the organism could be observed. Lactic dehydrogenase was so much the most active of the enzymes so far detected in *Agalactia* that it seemed possible that if this enzyme were inhibited the growth of the organism might be impaired. It was found that the addition of 1·0 % oxalate to the medium entirely prevented growth of *Agalactia*. Oxalate in lower concentrations permitted some growth, while concentrations of gluconate up to and including 1·0 % retarded but did not inhibit the growth of *Agalactia*. Subcultivation on

to normal medium gave normal growth. The addition of broth had a slight inhibitory effect on lactic dehydrogenase showing that under normal conditions of culture the enzyme cannot be optimally active. It seems unlikely that the concentration of any inhibitor occurring naturally will be great enough to inhibit growth.

The oxygen uptake of *Agalactia* in the presence of different substrates was measured in Barcroft differential manometers. Lactate, broth, serum, fructose and citrate were regularly oxidised while aspartate, alanine and succinate were irregularly attacked. In general it seemed that those substances which were dehydrogenated regularly in the presence of methylene blue could also be oxidised by oxygen while those which were only occasionally hydrogen donators to methylene blue were also only occasionally oxidised in air. The oxygen uptake of *Agalactia* in presence of broth was linear for the whole course of the experiment while the uptake in the presence of all other substrates fell off usually during the second hour. It was found that in presence of broth the enzymes remained active, while without it they became inactive during the shaking. The destruction of the enzymes was determined by testing the reducing capacity of the organism in the presence of fresh broth or lactate at the end of the experiment. *Agalactia* which has been shaken in the manometer with broth still decolorises methylene blue rapidly whereas after shaking in the presence of any other substrate the reduction of methylene blue does not occur. This protective effect of broth is unexplained.

SUMMARY.

A more comprehensive survey of the dehydrogenases and oxidising enzymes of *Agalactia* has been made. The most active dehydrogenases are those attacking lactate, adenylic acid, adenosine, fructose and hexosediphosphate. Other dehydrogenases need very high substrate concentrations. The relative strengths of the dehydrogenases vary from culture to culture. This variability does not seem to depend entirely on the medium or on the phase of growth at which the enzymes are estimated. The lactate enzyme is similar to the lactic dehydrogenase of *Esch. coli* in that it is strongly inhibited by gluconate and oxalate.

I have much pleasure in thanking the Medical Research Council for a personal grant and Mrs B. E. Holmes for assistance in this work. Also I wish to thank Mr P. J. G. Mann for some glucose dehydrogenase co-enzyme.

REFERENCES.

Bedson (1933). *Brit. J. Exp. Path.* **14**, 267.
Elford and Galloway (1933). *J. Path. Bact.* **37**, 381.
Ledingham (1933). *J. Path. Bact.* **37**, 393.
Mann (1932). *Biochem. J.* **26**, 785.
Quastel and Whetham (1924). *Biochem. J.* **18**, 519.
—— —— (1925). *Biochem. J.* **19**, 520.
Pirie and Holmes (1933). *Brit. J. Exp. Path.* **14**, 290.

LVIII. A FURTHER NOTE ON THE CHLORIDES AND INORGANIC CONSTITUENTS OF THE SERUM AND CEREBROSPINAL FLUID IN NEPHRITIS.

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(*Received February 13th, 1934.*)

Linder and Carmichael [1931] recorded the results of a study of the inorganic constituents of the serum and cerebrospinal fluid in four cases of advanced nephritis. They hoped to find an explanation for the very high level of the cerebrospinal fluid-chloride which has been found in some cases of uraemia. But cases of uraemia showing this feature are somewhat rare, for the highest they found over a period of some years was 761 mg./100 ml. This was in Case IV of the above paper, and the findings in that case are included in Table I, together with those in Hamilton's [1925] case Mc. Gn. which is the only other case of this kind for which data are available. In both of these the relationship of the chloride of the cerebrospinal fluid to the chloride of the serum and also that of the bicarbonate showed unusual departures from the normal. It was therefore suggested that a breakdown of the normal regulating mechanism was the condition which gave rise to the occasional very high chlorides in uraemia.

The following figures (Case V) were obtained from the arterial blood and cerebrospinal fluid of a man suspected of having meningitis. The discovery that the cerebrospinal fluid-chloride was 850 mg./100 ml. led to the diagnosis of uraemia. Even at rest dyspnoea was intense. Death occurred 12 hours later.

All methods employed were the same as those used by Linder and Carmichael [1931].

Table I.

	Blood-urea mg./100 ml.	Chloride: Serum-water (*mM*)	Chloride: CSF (*mM*)	Cl_{SW}/Cl_{CSF}	Bicarbonate: Serum-water (*mM*)	Bicarbonate: CSF (*mM*)	$HCO_{3\,SW}/HCO_{3\,CSF}$	Total base: Serum-water (*mM*)	Total base: CSF (*mM*)	B_{CSF}/B_{SW}	Inorganic phosphorus: Serum-water (*mM*)	Inorganic phosphorus: CSF (*mM*)	Protein: Serum (g./100 ml.)	Protein: CSF (g./100 ml.)	Other acids: Serum-water (*mM*)	Other acids: CSF (*mM*)
Mc.Gn.	—	118	142	0·83	16	16	1·0	178	174	0·98	—	1·3	6·8	—	—	—
Case IV	380	98	127	0·77	13·0	13·2	0·98	164	—	—	4·7	5	7·0	0·09	26	—
Case V	800	116	145	0·80	2·8	7·4	0·38	167·5	154·5	0·92	2·5	0·9	7·3	0·175	31	2

The points of interest in the results from Case V are (1) that the serum-water chloride has not risen in proportion to the cerebrospinal fluid-chloride, and therefore the ratio Cl_{SW}/Cl_{CSF} is 0·80; this is lower than any recorded ratio except that of Case IV; (2) that the acidosis is extreme, the bicarbonate being so low that the formula suggested by Hamilton correlating serum-water and cerebrospinal fluid-bicarbonates could not be applied; (3) that the total base

shows a normal distribution; (4) that the distribution of phosphorus and undetermined anions suggests that the meningeal barrier is able to resist the passage of these into the cerebrospinal fluid.

In Case IV the acidosis was less severe, but the normal regulating mechanism showed greater disturbance, for the bicarbonate was the same in the two fluids instead of being higher in the cerebrospinal fluid as Hamilton's formula would require; the phosphorus was the same instead of being lower in the cerebrospinal fluid, and the chloride ratio was reduced to 0·77. The conditions found by Hamilton in Mc. Gn. were similar to those in Case IV.

The hypothesis that the very high chloride in the cerebrospinal fluid is concerned with a departure from the normal relationship to the chloride of the serum is supported by these additional results. But it is evident that a second important factor is the level of the serum-water chloride. When this is high as in Case V and in Mc. Gn. the cerebrospinal fluid chlorides reach a higher level than in Case IV, although the disturbance of chloride equilibrium was greater in the last. A third factor may possibly be the retention by the meningeal barrier of the power to resist the invasion of the cerebrospinal fluid by phosphates, sulphates and other anions from the serum, thus preserving base to carry chloride and bicarbonate. Atchley and Benedict [1930] found that a decrease in serum-chlorides was the rule in uraemia, but Bulger *et al.* [1926] point out that in uraemia with high serum-chlorides the bicarbonate may be extremely low, which was so in Case V. These facts explain the rarity of very high cerebrospinal fluid-chlorides in uraemia and their evil significance.

SUMMARY.

A very high cerebrospinal fluid-chloride titre in uraemia indicates the presence of (i) a disturbance of the normal serum-cerebrospinal fluid relationships, and (ii) a high serum-chloride with a very severe acidosis.

My thanks are due to Dr J. F. Wicht, Medical Superintendent of the City Hospital, Capetown, for the opportunity to study this patient who was under his care, and to Prof. B. J. Ryrie for his interest in this investigation.

REFERENCES.

Atchley and Benedict (1930). *J. Clin. Invest.* **9**, 265.
Bulger, Peters, Eisenman and Lee (1926). *J. Clin. Invest.* **2**, 213.
Hamilton (1925). *J. Biol. Chem.* **65**, 101.
Linder and Carmichael (1931). *Biochem. J.* **25**, 1090.

LIX. THE EFFECT OF HALOGEN SALTS ON STEAPTIC DIGESTION.

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(*Received February 2nd, 1934.*)

Following on work as to the effect of halogen salts on salivary, peptic and tryptic digestion [Clifford, 1925; 1927; 1928; 1933], it was decided to carry out experiments on their action on lipase.

The effect of halogen salts on lipolytic activity has been investigated many times with varying results. Loevenhart and Pierce [1907], using pancreatic and hepatic lipases, found sodium fluoride strongly inhibitory, as also, though to a far less degree, were sodium chloride and iodide, potassium chloride and iodide and potassium bromide. Sodium bromide, however, was found by these observers to exert a slight accelerating action. Amberg and Loevenhart [1908] confirmed the inhibitory action of sodium fluoride on hydrolysis by hepatic lipase.

Mellanby and Woolley [1914] found no effect on lipolytic activity with the chlorides of sodium, calcium and magnesium, but Falk [1918] stated that sodium chloride accelerated the action over a period of 30 minutes. This acceleration disappeared if the fat were previously emulsified and was replaced by a retardation if the concentration of chloride were increased.

Leake *et al.* [1929] found that sodium fluoride in a concentration of 1 : 50,000 inhibited hepatic lipase *in vitro* and extended the work to observations *in vivo*, obtaining similar results. In the same year Corran [1929] found the chlorides of sodium and potassium to be slightly augmentative, and calcium chloride without effect. McGillivray [1930] confirmed these observations for sodium chloride but stated that calcium chloride also hastened lipase action.

These results, except for agreement as to the inhibitory action of sodium fluoride, vary considerably, and it was decided to investigate the action of various concentrations of halogen salts on the rate of lipolytic activity.

Experimental methods.

Since in the digestion of fats emulsification is obviously of great importance, the substrate used was the relatively stable emulsion found naturally in cow's milk.

A solution of commercial pancreas substance was used as the source of lipase, since in a low concentration it showed marked lipolytic activity together with a negligible tryptic action. A solution of the pancreas substance in the concentration used in these experiments showed no proteolytic action (biuret reaction unchanged in each case and this negative result confirmed by Sørensen titrations with fish-protein) in 5 hours, when incubated together with sodium carbonate at 37° with caseinogen, fish-protein or hard-boiled egg-white.

In any set of experiments 200 ml. of milk were placed in a flask together with the requisite weighed amount of halogen salt. The flasks were allowed to reach a

temperature of 37° in a water-bath, when 20 ml. of a 0·5 % solution of pancreas substance also warmed to 37° were added; 25 ml. of this mixture were removed immediately, about 150 ml. of boiling water added to stop enzyme action, and the liquid was titrated against 0·1 *N* NaOH using phenolphthalein as an indicator.

Further 25 ml. portions were removed at 15 minute intervals and treated similarly.

The milk used had been pasteurised and gave a negligible rise in acidity when kept for 2 hours at 37°.

A typical experimental result is as follows:

200 ml. milk with 0·125 *M* LiBr.

20 ml. 0·5 % pancreas substance.

25 ml. removed at 15 minute intervals and titrated with 0·1 *N* NaOH.

Minutes	ml. 0·1 *N* NaOH	ml. 0·1 *N* acid produced
0	3·6	—
15	6·8	3·2
30	8·9	5·3
45	11·7	8·1
60	13·6	10·0
75	13·8	10·2
90	14·7	11·1
105	14·5	11·2

In each series two control flasks were used and one of each of the halogen mixtures. The concentrations investigated varied from 0·5 to 0·008 *M*. Three separate batches of pancreas substance were used and at least a similar number of samples of each halogen salt.

Results.

Fluorides. Typical results with fluorides are given in Table I. From them it can be seen that sodium, potassium and ammonium fluorides are strongly

Table I. *Fluorides.*

ml. 0·1 *N* acid produced.

Time of digestion in minutes	15	30	45	60	75	90	105	15	30	45	60	75	90	105
				0·5 *M*							0·25 *M*			
Control A	0·3	2·4	4·6	6·2	7·2	8·3	8·9	0·9	2·1	4·0	5·0	6·0	6·6	7·4
,, B	0·8	3·0	5·0	6·4	7·4	8·9	9·1	0·5	2·4	3·8	4·8	5·6	7·0	7·1
Na	0	0	0·7	1·5	1·9	2·1	2·3	0	0	0·1	0·3	0·5	0·5	0·6
K	0	0	0	0·4	1·0	1·5	2·1	0·1	0·4	1·0	1·2	1·7	1·7	2·1
NH_4	0	0	0	0·1	0·5	1·6	2·1	0	0	0·1	0·1	0·1	0·2	0·7
				0·125 *M*							0·06 *M*			
Control A	2·1	4·6	6·4	7·9	9·1	10·7	11·0	2·1	4·8	6·4	8·2	8·9	9·7	10·6
,, B	2·0	4·4	6·3	7·8	8·7	10·5	10·9	2·5	4·7	6·3	8·0	8·7	10·2	10·4
Na	1·0	2·6	3·6	4·2	4·4	4·9	5·7	1·3	2·5	3·5	4·8	5·7	6·3	7·6
K	0·8	2·5	3·6	3·8	4·3	4·5	5·5	0·9	2·7	3·2	3·8	4·8	5·6	6·0
NH_4	0·6	0·8	0·9	1·4	1·4	1·6	2·4	0·2	0·4	1·3	2·5	3·7	4·5	4·6
				0·03 *M*							0·015 *M*			
Control A	2·9	4·8	7·6	9·0	9·9	10·3	11·2	2·2	5·3	6·9	8·6	9·2	10·2	11·3
,, B	3·0	5·3	7·2	8·8	9·9	10·8	11·0	2·5	5·2	7·1	8·5	9·5	9·9	11·2
Na	2·1	4·0	5·0	6·4	7·1	7·4	8·7	1·7	4·5	5·4	6·2	7·3	8·9	9·0
K	2·2	3·9	5·7	7·7	7·9	8·6	8·9	1·7	4·4	5·7	7·0	7·5	7·8	9·0
NH_4	2·3	4·2	5·8	6·8	7·6	8·6	8·9	1·1	4·2	5·8	6·9	7·6	8·1	9·2
				0·008 *M*										
Control A	0·3	2·4	4·3	4·5	5·9	6·9	8·9							
,, B	2·4	3·0	4·1	4·4	5·6	6·8	8·3							
Na	1·1	1·8	2·6	3·2	3·6	5·0	6·5							
K	0·6	1·2	2·4	2·6	3·5	4·4	5·4							
NH_4	1·4	1·8	3·2	3·8	4·2	4·7	5·9							

Table II. *Chlorides.*

ml. 0·1 *N* acid produced.

Time of digestion in minutes	15	30	45	60	75	90	105	15	30	45	60	75	90	105
				0·5 *M*							0·25 *M*			
Control A	1·2	3·3	6·3	8·4	8·7	9·6	10·0	2·3	4·5	6·2	7·1	9·1	9·7	10·6
,, B	1·4	4·5	5·9	8·3	8·9	9·5	10·1	2·0	4·4	5·9	7·0	9·2	9·9	10·4
Li	1·2	4·8	6·0	7·8	9·0	9·9	10·3	1·6	4·4	6·0	7·9	9·1	9·4	9·9
Na	0·9	4·5	6·6	8·0	8·3	8·9	9·5	1·4	4·6	5·8	7·1	9·3	9·5	10·0
K	0·8	4·7	5·8	8·2	8·8	9·2	9·7	1·7	3·8	5·7	7·9	9·0	9·9	10·0
NH_4	0·8	3·6	6·3	7·5	8·6	9·0	9·6	1·8	4·5	5·2	7·3	9·7	10·0	10·4
				0·125 *M*							0·06 *M*			
Control A	2·1	4·9	7·1	8·8	10·6	11·1	12·0	1·7	3·4	5·6	7·7	8·5	9·7	10·4
,, B	2·3	4·4	6·2	7·8	10·8	10·8	11·4	1·5	3·0	5·3	7·4	8·3	9·1	10·7
Li	2·1	4·5	6·7	7·3	9·6	10·2	12·1	1·8	2·9	5·5	7·5	8·0	8·9	10·2
Na	1·9	5·0	7·6	8·6	10·3	11·4	11·5	1·5	3·1	5·4	7·6	8·4	9·3	10·5
K	1·4	4·7	6·4	7·9	10·0	10·7	12·0	1·6	3·5	5·2	7·7	8·2	9·5	10·6
NH_4	1·8	4·2	6·1	7·3	9·4	11·3	11·3	1·8	3·3	5·5	7·6	8·4	9·4	10·3
				0·03 *M*							0·015 *M*			
Control A	2·5	4·7	6·3	8·0	8·7	10·2	10·4	3·3	6·6	8·4	9·5	11·9	12·2	12·3
,, B	2·0	4·5	6·1	8·2	8·9	10·3	10·6	3·0	6·4	8·5	9·8	11·7	12·0	12·5
Li	1·9	4·0	5·9	8·0	8·7	10·4	10·7	3·1	6·3	8·4	9·6	11·6	11·9	12·2
Na	2·3	4·4	6·0	8·4	8·8	10·1	10·5	3·3	6·5	8·5	9·7	11·5	12·1	12·4
K	2·4	4·3	6·2	7·9	8·5	10·4	10·6	3·1	6·2	8·6	9·4	11·3	12·2	12·4
NH_4	2·2	4·4	6·4	8·0	8·6	9·9	10·3	3·2	6·6	8·5	9·5	11·5	12·3	12·5
				0·008 *M*										
Control A	1·9	3·4	6·5	7·7	9·5	10·7	11·6							
,, B	2·0	3·5	6·7	7·9	9·8	10·8	11·9							
Li	1·9	3·3	6·4	7·8	9·7	10·7	11·5							
Na	2·1	3·5	6·6	7·5	9·6	10·6	11·7							
K	2·3	3·6	6·6	7·7	9·8	10·8	11·5							
NH_4	2·0	3·4	6·5	7·6	9·7	10·7	11·8							

Table III. *Bromides.*

ml. 0·1 *N* acid produced.

Time of digestion in minutes	15	30	45	60	75	90	105	15	30	45	60	75	90	105
				0·5 *M*							0·25 *M*			
Control A	1·0	2·5	4·3	5·8	6·6	7·5	7·5	1·3	5·0	7·0	9·2	9·7	10·1	12·3
,, B	1·0	2·6	4·3	6·3	6·6	7·2	7·6	1·1	4·9	6·8	9·0	9·5	9·9	11·8
Li	0·3	1·9	3·9	4·3	5·1	5·7	6·1	0·7	4·5	7·2	8·5	9·2	9·4	11·2
Na	0·2	1·5	3·3	4·5	5·1	6·7	6·7	0·9	3·4	6·7	7·7	8·6	9·6	11·5
K	0·5	1·9	3·5	4·6	5·3	5·8	6·6	0·5	3·4	6·6	8·6	9·0	9·8	10·8
NH_4	0·3	1·1	3·7	5·0	5·2	5·6	6·5	3·1	4·3	7·7	8·4	8·6	9·4	11·7
				0·125 *M*							0·06 *M*			
Control A	2·2	5·3	6·9	8·6	9·2	10·2	11·3	1·4	3·4	6·5	8·6	8·9	9·9	10·2
,, B	2·3	5·4	7·0	8·3	9·3	10·0	11·1	1·5	3·7	6·4	8·5	9·1	9·8	10·3
Li	2·0	5·5	7·1	8·5	9·1	10·3	11·0	1·5	3·6	6·6	8·4	9·0	10·0	10·4
Na	2·1	5·4	7·0	8·4	9·4	10·2	11·2	1·3	3·5	6·4	8·5	8·9	10·1	10·3
K	2·3	5·6	7·2	8·5	9·2	10·1	11·3	1·2	3·4	6·3	8·6	9·2	9·9	10·3
NH_4	2·2	5·1	7·2	8·6	9·5	10·4	11·4	1·5	3·8	6·6	8·7	9·0	10·1	10·4
				0·03 *M*							0·015 *M*			
Control A	2·0	4·8	7·1	8·6	10·4	11·0	11·9	2·3	4·3	7·2	8·9	9·7	10·5	11·0
,, B	2·1	4·0	6·0	7·6	10·7	11·0	11·5	2·0	4·7	7·0	8·9	9·9	10·4	10·9
Li	2·0	4·3	6·5	7·1	10·3	10·9	11·8	2·1	4·3	7·5	9·1	9·8	10·6	10·8
Na	1·9	4·7	7·2	8·4	10·5	11·2	11·7	2·1	4·5	6·9	9·3	9·8	10·5	10·7
K	1·6	4·8	6·5	8·3	10·4	11·1	11·7	2·2	4·7	7·4	9·2	10·0	10·6	11·1
NH_4	2·1	4·6	6·5	7·7	10·6	11·5	11·6	1·9	4·4	7·1	8·9	9·7	10·5	10·9
				0·008 *M*										
Control A	1·7	2·9	5·0	7·5	9·4	10·0	10·5							
,, B	1·3	2·8	5·0	7·4	9·2	9·8	10·1							
Li	1·5	2·7	4·9	7·3	9·0	9·9	10·4							
Na	1·8	3·4	5·3	7·5	8·9	9·7	10·5							
K	1·4	2·9	5·1	7·6	9·0	9·8	10·3							
NH_4	1·5	2·8	5·3	7·5	9·4	9·9	10·4							

Table IV. *Iodides.*

Time of digestion in minutes	ml. 0·1 *N* acid produced.													
	15	30	45	60	75	90	105	15	30	45	60	75	90	105
	0·5 *M*							0·25 *M*						
Control A	3·1	5·7	7·9	8·5	9·7	10·4	10·7	1·3	2·4	4·6	6·2	7·2	8·3	8·9
,, B	3·2	5·3	7·8	8·4	9·2	9·9	10·0	1·8	3·0	5·0	6·4	7·4	8·9	9·4
Li	2·7	5·2	7·3	7·6	9·0	9·4	10·4	0·9	2·5	4·7	6·0	7·1	8·5	9·3
Na	3·6	6·5	8·7	8·9	9·7	10·4	10·6	1·1	2·7	4·8	6·3	7·3	8·5	9·2
K	3·5	5·3	8·1	8·6	9·2	10·7	10·9	1·3	2·3	4·5	6·3	7·4	8·6	9·2
NH_4	3·1	5·5	8·3	8·5	9·7	10·7	11·2	1·2	2·6	4·9	6·4	7·5	8·9	9·4
	0·125 *M*							0·06 *M*						
Control A	0·8	2·0	3·1	4·9	5·2	6·1	6·9	2·1	4·4	6·2	7·7	9·0	10·6	10·9
,, B	0·7	2·1	3·4	4·8	5·9	6·5	7·1	2·0	4·2	6·1	7·9	8·9	10·5	11·0
Li	1·5	2·8	4·2	5·5	5·7	6·7	7·0	1·9	4·0	6·3	8·0	9·1	10·7	11·2
Na	0·8	2·4	3·5	4·9	5·4	6·2	7·4	2·0	4·4	6·4	7·8	9·0	10·4	10·8
K	0·5	2·2	3·1	4·9	5·8	6·6	7·2	2·1	4·3	6·4	7·6	9·1	10·6	11·0
NH_4	0·6	2·5	3·2	4·7	5·6	6·5	7·1	1·9	4·2	6·5	7·8	8·9	10·6	11·1
	0·03 *M*							0·015 *M*						
Control A	2·5	4·4	7·5	9·0	9·7	10·5	11·2	2·9	5·2	7·5	8·0	9·2	10·1	10·7
,, B	2·3	4·7	7·3	8·9	9·5	10·2	10·7	3·0	5·3	7·8	8·2	9·0	9·9	10·4
Li	2·3	4·5	7·9	9·1	9·9	10·4	11·0	2·8	5·2	7·4	8·3	9·3	10·2	10·6
Na	2·1	4·3	7·4	9·0	9·8	10·5	10·8	2·9	5·5	7·9	8·1	9·2	10·4	10·6
K	2·2	4·7	7·8	9·2	10·0	10·7	11·1	3·1	5·6	8·0	8·6	9·2	10·1	10·8
NH_4	2·0	4·4	7·8	9·5	9·9	10·5	10·9	2·9	5·5	7·8	8·5	9·1	10·4	10·9
	0·008 *M*													
Control A	2·0	5·0	7·1	8·5	10·4	10·9	11·5							
,, B	2·3	4·6	6·9	8·8	10·5	11·0	11·4							
Li	2·1	4·7	6·7	8·3	10·5	10·8	11·6							
Na	2·0	4·9	7·1	8·6	10·3	11·1	11·7							
K	2·2	4·7	6·8	8·4	10·4	10·9	11·6							
NH_4	1·9	4·5	6·6	8·4	10·6	11·3	11·7							

inhibitory in all concentrations from 0·5 to 0·06 *M* and definitely so even at a concentration of 0·008 *M*.

In Fig. 1 a series of experiments with sodium fluoride in varying concentrations shows how the inhibitory action is a function of concentration. Ammonium fluoride is more inhibitory than either sodium or potassium fluoride up to a concentration of 0·03 *M*. Below this strength all three salts have an equal effect.

Chlorides (Table II). In concentrations of 0·5–0·008 *M* the chlorides of lithium, sodium, potassium and ammonium have no effect on the rate of lipolytic action.

Bromides (Table III). With a concentration of 0·5 *M* there is a slight depression of rate, but from 0·25 to 0·008 *M* no effect can be demonstrated.

Iodides (Table IV). As with chlorides, there appears to be no acceleration or retardation of lipolytic action when iodides of lithium, sodium, potassium or ammonium are added in concentrations varying from 0·5 to 0·008 *M*.

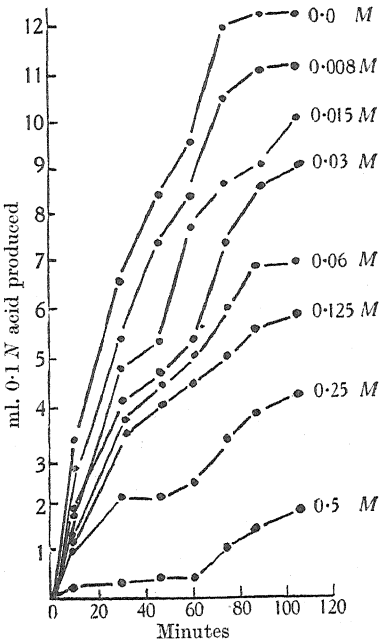

Fig. 1. Sodium fluoride 0–0·5 *M*.

Discussion.

From these results it appears that the halogen salts of lithium, sodium, potassium and ammonium are without effect on the rate of lipase action in concentrations

of 0·5–0·008 *M*, except in the case of the fluorides. Fluorides exert a strongly depressant action which varies directly with concentration of salt. Ammonium fluoride at 0·25–0·06 *M* is more inhibitory than either the sodium or potassium salt, but at 0·5 *M* and from 0·3 to 0·008 *M* all three fluorides show an equal effect.

During the course of these experiments an unexpected effect of halogen salts on the clotting of milk by very weak trypsin was observed. Though the proteolytic enzyme present with the lipase was too weak to show any digestive action on fish-protein, caseinogen or hard-boiled egg-white in five hours, yet in all the concentrations of ammonium fluoride, a junket was formed 15 minutes after the addition of 20 ml. of pancreas substance to 200 ml. milk.

Similar thick soft clots were formed with all four salts of chlorine, bromine and iodine and with potassium and sodium fluorides, except that at the lowest concentration (0·008 *M*) a fine flocculation rather than complete coagulation took place. From these results it seems that the coagulating effect of added calcium salts when rennin, pepsin or trypsin is added to milk may be due to the effect of the acid radical as well as to the metallic ion.

Further experiments on this coagulating action of the halogen salts are in progress.

Summary.

1. The lipolytic activity of pancreas substance is strongly inhibited by sodium, potassium and ammonium fluorides.

2. This inhibition varies directly with the concentration of fluoride.

3. Sodium, potassium and ammonium chlorides and iodides show no effect on the rate of lipase action in concentrations of 0·5–0·008 *M*.

4. Sodium, potassium and ammonium bromides depress lipolytic activity to a slight extent at a concentration of 0·5 *M* but are without effect at 0·25–0·008 *M*.

5. None of the halogen salts, in the concentrations used (0·5 – 0·008 *M*), accelerated lipolytic action.

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REFERENCES.

Amberg and Loevenhart (1908). *J. Biol. Chem.* **4**, 149.
Clifford (1925). *Biochem. J.* **19**, 218.
—— (1927). *Biochem. J.* **21**, 544.
—— (1928). *Biochem. J.* **22**, 1128.
—— (1933). *Biochem. J.* **27**, 326.
Corran (1929). *Biochem. J.* **23**, 188.
Falk (1918). *J. Biol. Chem.* **36**, 229.
Leake, Dalmes, Treweek and Loevenhart (1929). *Amer. J. Physiol.* **90**, 426.
Loevenhart and Pierce (1907). *J. Biol. Chem.* **2**, 397.
McGillivray (1930). *Biochem. J.* **24**, 891.
Mellanby and Woolley (1914). *J. Physiol.* **48**, 247.

LX. A NOTE ON THE INFLUENCE OF p_H ON THE ACTION OF HAEMOLYTIC COMPLEMENT.

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(*Received February 15th, 1934.*)

This problem, which is fundamental to any work on haemolytic complement, does not appear to have received the attention which it deserves. Fuchs [1930] showed that bubbling carbon dioxide through plasma for a short time (6 minutes) increased its complement activity, while bubbling for a longer time (30 minutes) decreased it. He found that the action was increased by carbon dioxide, as long as the reaction was alkaline or neutral to litmus and that it was reduced immediately it became acid to that indicator. It is now thought that CO_2 may have a specific effect on complement, *i.e.* an effect other than through p_H. Andreesco [1930] found that anticomplementary sera had a higher alkaline reserve than normal. This may conceivably be the p_H effect.

Michaelis and Skwirsky [1910] used a series of mixtures of $N/7$ primary and secondary phosphates of sodium and found that specific haemolysis had its optimum at a slightly alkaline reaction corresponding with that of blood. An increase in alkalinity retards haemolysis but not to the extent that the increase in acidity does. Eagle and Brewer [1929] varied the reaction of the medium by the addtion of $N/7$ NaOH and $N/7$ HCl. They found the optimum reaction for haemolysis to be between p_H 6·5 and 8. At p_H 5·3 and in the alkaline range there is an irreversible inactivation, complete at 4·8 and 8·8 respectively.

Unpublished observations indicate that relatively small variations in osmotic pressure affect the speed of complement action. It was therefore decided to repeat these observations with a stricter control of the osmotic pressure of the solution than that of previous workers. A method described by the author was used [Osborn, 1933]. This gives greater accuracy than the serial tube method, used by other observers in this field. It should be noted that this method measures the speed of complement action, whereas the former measures the end results of varying volumes of serum.

In earlier observations an isotonic phosphate buffer was used following Sørensen's method [1909]: this was discarded in favour of a mixture giving a wider range of p_H, *viz.* the "universal buffer mixture" of Prideaux and Ward [1924], as supplied by the B.D.H. Ltd. This mixture—consisting of phosphoric, boric and phenylacetic acids—was made up in twice the concentration recommended by the formula, adjusted to the required p_H by the addition of $0{\cdot}4N$ NaOH (this too, being twice the formula strength) and adjusted to approximately the osmotic pressure of corpuscles by the addition of water. Determinations of p_H were made by means of a double hydrogen electrode and of osmotic pressure by measurement of the depression of freezing-point, all solutions being adjusted to within 0·02 % of 0·9 % NaCl.

In determining the speed of complement action $(3{\cdot}5-x)$ volume of buffer, x volumes of diluted serum and 0·5 volume of a 3 % suspension of sheep's

corpuscles sensitised with 5 units of amboceptor were mixed and placed in a bath at 37°, and the time for the complete haemolysis of the corpuscles was measured. Several different samples of guinea-pig's serum were used in varying concentrations. The guinea-pig's serum and the sheep's corpuscles were supplied by the S.A. Institute for Medical Research; the details of the preparation of the latter have been described previously [Osborn, 1933].

Examples of the results obtained, giving the extreme cases, are recorded graphically in Fig. 1. It will be seen that the optimum p_H is in the region of 7·5

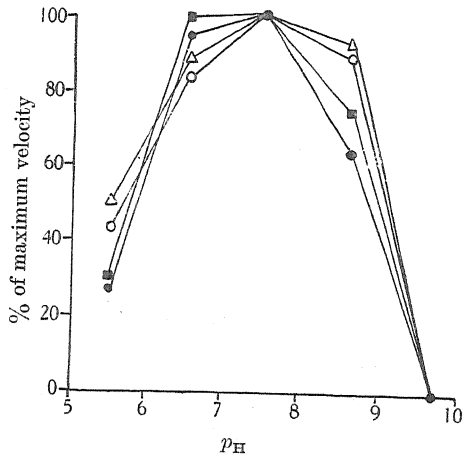

Fig. 1. Showing variation of speed of complement action with p_H.

and that the actual position of the optimum point and the exact shape of the curve vary in different sera, but agree in showing some degree of slowing between p_H 6 and 7, and a marked slowing in the region of p_H 5·5 on the acid side, and a slowing between p_H 8 and 9 and complete inhibition at p_H 9·7 on the alkaline side. These results are in accordance with those of Michaelis and Skwirsky [1910] and Eagle and Brewer [1929]. It is unlikely that more consistent results will be obtained, until it is possible to separate the complement complex from the other constituents of the serum.

Several interesting facts were observed in the course of this work. The buffers themselves retarded haemolysis, for the optimum time with a buffer was at least twice that with normal saline. This retarding influence is common to a wide variety of inorganic and organic ions. (Unpublished observations.)

An attempt to investigate the alkaline range with borate buffers was frustrated by the fact that this ion may be haemolytic to both sensitised and unsensitised corpuscles. The effect appears to be roughly proportional to the hydrogen ion concentration and disappears at about p_H 8. The presence of sodium chloride and other ions protects the corpuscle from this haemolysis.

The observation of Coulter [1921], that heat-inactivation is most rapid in alkaline medium, was confirmed. The haemolytic activity of serum standing at room temperature for 24 minutes at p_H 8·6 was reduced by 44 %, while it suffered no demonstrable change at p_H 7·5, 6·5 or 5·5. That this was a true heat-inactivation was shown by the fact that a control at p_H 8·6 standing in ice-water showed no change.

The author wishes to express his indebtedness to Dr Buchanan of the S.A. Institute for Medical Research for the supply of guinea-pig's serum and sensitised sheep's corpuscles.

REFERENCES.

Andreesco (1930). *Compt. Rend. Soc. Biol.* **104**, 937.
Coulter (1921). *J. Gen. Physiol.* **3**, 771.
Eagle and Brewer (1929). *J. Gen. Physiol.* **12**, 845.
Fuchs (1930). *Z. Immunitätsforsch.* **67**, 266.
Michaelis and Skwirsky (1910). *Z. Immunitätsforsch.* **4**, 357.
Osborn (1933). *Biochem. J.* **27**, 1425.
Prideaux and Ward (1924). *J. Chem. Soc.* **125**, 426.
Sørensen (1909). *Biochem. Z.* **21**, 131.

LXI. THE ACTION OF THE CINCHONA AND CERTAIN OTHER ALKALOIDS IN BIRD MALARIA. II.

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(*Received February 16th, 1934.*)

In the previous paper [Goodson *et al.*, 1930], it was shown that Roehl's method of investigating the relative therapeutic efficiencies of antimalarial drugs, by trial in bird malaria, might prove sufficiently delicate to show quantitative differences in action between the eight principal alkaloids of cinchona bark, *viz.* *l*-quinine, *l*-cinchonidine, *d*-quinidine, *d*-cinchonine (which may conveniently be called the primary cinchona alkaloids) and the reduction products yielded by each of these four bases. In these reduction products the vinyl side-chain of each parent alkaloid is reduced to an ethyl group: they are therefore dihydro-derivatives and are so named throughout this paper. Commercially they are known as hydroquinine, hydrocinchonine, *etc.*

The determination of the relative antimalarial values of the several cinchona alkaloids has now assumed greater practical importance owing to the introduction, by the Health Organisation of the League of Nations, of "totaquina," a product designed as an economical substitute for quinine in the mass treatment of indigent, malarial populations. "Totaquina" is defined [Malaria Commission, League of Nations, 1931], as a mixture of cinchona alkaloids containing a minimum of 15 % of quinine and 70 % of crystallisable cinchona alkaloids, with a maximum of 20 % of amorphous cinchona alkaloids, 5 % of moisture and 5 % of ash.

This innovation, based on the view that there is not a great deal to choose between the crystalline cinchona alkaloids as antimalarial drugs, does not meet with universal acceptance among malariologists, but the opinion of the majority is probably not unfairly represented by the following quotation:

"There is no conclusive evidence to show that any one of the crystallisable alkaloids is so markedly superior to any other as to justify its exclusive use in ordinary cases of malaria in preference to any other, taking into account the shortage of these alkaloids and their cost" [Sinton, 1930].

The misgivings aroused by proposals to substitute for quinine any one of the secondary cinchona alkaloids, or of mixtures such as "totaquina," may be indicated by the following quotation:

"En *premier* lieu il était nécessaire de démontrer que la malaria *peut* être guérie au moyen des alcaloïdes mineurs. Nous n'avons pas besoin d'insister sur ce point qui est, pensons nous, universellement admis.

"En *second* lieu il était nécessaire de donner des préceptes nets au sujet de la façon dont ces alcaloïdes doivent être appliqués pour donner le degré de sécurité auquel on était habitué par un emploi pendant presqu'un siècle de la quinine. Il fallait établir (*a*) à quelles exigences de pureté les nouvelles préparations à

introduire doivent satisfaire et quelle doit être leur composition et (*b*) quelle est la dose à laquelle il faut les administrer pour le succès soit assuré" [Van der Sleen and Drossart Lulofs, 1931].

The definition of "totaquina" allows considerable latitude in the relative proportions of the various cinchona alkaloids present, and in consequence there is much variation in this respect in commercial "totaquina," as the following analyses show:

Table I. *Composition of "totaquina."*

	Type I % *	Type II % †	Type III % *
Quinine	32·5	15·3	17·0
Cinchonidine	34·2	5·7	24·0
Quinidine	—	5·2	10·0
Cinchonine	20·1	55·4	23·0
Total crystallisable alkaloids	86·8	81·6	74·0
Amorphous alkaloids	9·9	13·4	17·0
Moisture	2·6	1·9	5·0
Ash	0·2	2·1	4·0

* Manufacturer's analysis. † Analysis by Mr J. A. Goodson.

It is obvious that if there is a marked difference in the action of, say quinine and cinchonine, the therapeutic values of "totaquina" of types I and II will be different and some evidence for this view has already been brought forward as a result of trials of these two types in bird malaria [Giemsa, 1932] and in human malaria [Malaria Commission, League of Nations, 1933].

It appeared to the authors that definite quantitative evidence on this matter could best be obtained by comparative trials of pure specimens of the individual alkaloids in bird malaria.

The four primary cinchona alkaloids have been purified by processes described later, and a method of controlling these processes, so far as the elimination of the dihydro-bases is concerned has been devised. From the primary bases the pure dihydro-bases have been prepared by catalytic hydrogenation. As there was no certainty that any of these eight alkaloids had been obtained pure previously the principal constants of each have been determined and are recorded and compared with the best of the known previous determinations in Tables II, III and IV.

The biological trials gave results which are summarised in Table A. The figures represent the dose of quinine necessary to produce the same amount of protection as unit dose of the other alkaloids. The figures are only approximate, but the authors think that (*a*) the distinction between quinine and dihydroquinine is certain, (*b*) that dihydroquinidine, cinchonidine and quinidine are definitely less active than quinine and (*c*) that dihydrocinchonine and dihydrocinchonidine form a group of much lower activity than any of the others. The samples of quinidine and cinchonine used were not the purest available; the quinidine contained 1·8 % of dihydroquinidine and cinchonine 1·4 % of dihydrocinchonine. Trials with pure alkaloids were spoilt by the outbreak of a *B. gaertner* epidemic in the stock of canaries, but it will be seen that the removal of these amounts of the dihydro-alkaloids would not affect the order of activity given in the table.

It is not claimed that the order in which the drugs are arranged in this table necessarily represents the order of antimalarial activity in man. But it is logical to assume that, if the alkaloids differ so much in their activity on the parasite experimented with, they will not be of equal therapeutic value in man.

The final decision as to which is the most active must rest on clinical evidence, which is notoriously difficult to assess. It is however, with the present evidence, unjustifiable to assume that all the crystallisable alkaloids are of equal value.

The same order can be deduced from the results of previous workers [Goodson *et al.*, 1930; Giemsa *et al.*, 1926; Giemsa and Oesterlin, 1933] except as regards quinidine. Goodson *et al.* [1930] obtained results, which suggest that quinidine has a higher activity than that now assigned to it. The number of birds on which quinidine is given its place, is small, owing to the fact that some died during the experiment. It is probable that 5 mg. daily is too toxic a dose for quinidine, though the large majority of canaries survive these doses of the other alkaloids. In this respect our canaries are less sensitive than those used by Giemsa. The apparent difference cannot be explained by any difference in purity of the alkaloids used. The point is however being further investigated together with the proper place of cinchonine.

The effect of hydrogenation is astonishingly different for different alkaloids. For quinine and possibly quinidine, hydrogenation increases the activity; for cinchonine the effect is probably to diminish the activity. No possible explanation can be offered and yet another instance of lack of correspondence between biological activity and chemical structure must be left on record.

Table A.

Dihydroquinine	1–2	Quinidine	About 0·5*
Quinine	1	Cinchonine	Less than 0·2
Dihydroquinidine	0·5–1	Dihydrocinchonine	,,
Cinchonidine	About 0·5	Dihydrocinchonidine	,,

* Further experiments with the pure alkaloids suggest that this is an overestimate rather than an underestimate of the relative activity of quinidine.

Modified cinchona alkaloids. In the previous paper it was shown that although quitenine is itself inactive it recovers antimalarial activity on esterification; this activity is slight for the methyl and ethyl esters and only becomes well-marked in the butyl and amyl esters. Observations with two of these esters have been repeated with the results recorded in Table B, which are given in a form readily comparable with those of Goodson *et al.*

These results are interesting in two ways; (1) they are both positive, whereas in the former more extensive series negative results were occasionally obtained with each substance; (2) the improvement in activity from ethyl to amyl is not so well-marked as in the previous experiments. These alkylquitenines are somewhat readily hydrolysed, and it is suggested that the apparent variation in antimalarial activity is caused by varying capacity for hydrolysing the drugs shown by different canaries. Less variable results would probably be obtained by intravenous injection, a method not well suited for use with canaries.

Certain of the cinchona alkaloid "chlorides" being available for other purposes, the opportunity was taken to try them in bird malaria. Quinine chloride, dihydroquinine chloride and cinchonidine chloride all proved inactive in six daily doses of 5 mg. as was to be expected from the recorded inactivity of quinine chloride [Giemsa *et al.*, 1926]. It is however of interest to note that whilst in the authors' experiments no difficulty was experienced in giving doses of 5 mg. of these chlorides, Giemsa was only able to give 1·5 mg., a further example of variability in canaries. The disappearance of activity as a result of replacing the —CHOH— group by —CHCl— is a striking example of the important part played by this group in this set of alkaloids, activity disappearing

on its conversion into —CO— (quininone), —CH_2— (desoxyquinine), :CH— (quinene) or —CH.OAc— (acetyldihydroquinine) [Giemsa and Oesterlin, 1933] and no change so far effected has left the activity unimpaired.

Table B.

Substance	Daily doses: No.	Daily doses: Quantity mg.	Retardation days
Ethylquitenine hydrochloride	6	5	5
iso-Amylquitenine dihydrochloride	6	5	6

The authors desire to place on record their indebtedness to Prof. van der Sleen of the Kina Bureau, Amsterdam, for information on a number of technical points connected with the analysis of cinchona alkaloids. They also owe thanks to Mr W. Solomon, B.Sc., for the preparation of the three cinchona alkaloid chlorides mentioned, to Messrs A. C. Camfield, E. M. Gibbs, and N. J. G. McLaren for much patient work on the fractionation and biological tests of the alkaloids, and to Messrs A. Bennett and H. C. Clarke for the micro-analyses.

Chemical section.

Crystallisable cinchona alkaloids. The impurities in commercial samples of the primary cinchona alkaloids are of two kinds. Each alkaloid contains its own reduction product, which in the case of quinidine is now shown to be present to the extent of 20 % and may be as high as 30 % [Dawson and Garbade, 1930], while cinchonine usually contains about 14 % of dihydrocinchonine. These remarks apply to well-crystallised specimens of these alkaloids, usually accepted by chemists and biologists as pure owing to their constancy of melting-point and optical rotation. The amounts of dihydro-bases in quinine and cinchonidine are much less but are not negligible. The other type of impurity is due to the difficulty of completely separating the components of each of the two similarly optically active pairs from each other, *i.e.* cinchonidine and quinine usually contaminate each other, as do quinidine and cinchonine, but components of the laevorotatory pair are not usually found in those of the dextrorotatory pair and the converse is also true.

The purification of any one of the four primary alkaloids thus involves two steps: (1) the removal of the second component of the similarly optically active pair, *e.g.* cinchonidine from quinine and *vice versa*, and (2) the elimination from each alkaloid of the dihydro-base, which naturally accompanies it.

Much work has been expended in attempts to prepare each of the primary cinchona alkaloids in a pure state, but only two of the numerous papers on this subject need be referred to here [Tutin, 1909; Emde, 1932]. Tutin prepared pure quinine by repeated crystallisation of the acid sulphate, but was unable to prove that it was free from dihydroquinine. This proof is now supplied. Emde set out to prepare all eight alkaloids in a pure state, specially recommending for this purpose crystallisation of the dihydrobromides, but he did not prove the absence of the dihydro-bases from his final specimens of the four primary alkaloids.

In the course of the present investigation, pure specimens of each of the primary alkaloids have been prepared, the chief criteria of purity adopted (apart from well-known specific tests, such as fluorescence, methoxyl determinations and the thalleioquin reaction) being (*a*) agreement of the "found" and "calculated" values for hydrogen absorbed, by the conversion of the vinyl group into

an ethyl group, under standard, quantitative conditions, as proof of the absence of dihydro-bases and (*b*) constancy of optical rotation of the base and its salts, when expressed on a uniform basis and taken under standard conditions. The chief effects of this purification are to remove quinine from "cinchonidine" and quinidine from "cinchonine," and to eliminate large quantities of dihydro-bases from what have hitherto been commonly regarded as pure cinchonine and pure quinidine. From the pure primary alkaloids the corresponding dihydro-bases have been made by catalytic hydrogenation.

The chief constants of the eight pure alkaloids so prepared are recorded in Tables III and IV.

Though numerous determinations of the specific rotations of the cinchona alkaloids are recorded, they are for such varied concentrations and solvents, that it is not practicable to use them for comparative purposes. The authors have however repeated their determinations for the dihydrobromides of all eight alkaloids, under the conditions used by Emde in order to establish a more exact comparison with the results of that author. These comparative results are given in Table II.

Table II. *Specific rotations of dihydrobromides of cinchona alkaloids.*

Base	Salt used	*c* (anhydrous salt)	Solvent	$[\alpha]_D$ recorded by Emde	$[\alpha]_D$ recorded by Present authors
Quinine	B.2HBr, $3H_2O$	3·5194	Water	−181·04°*	−184·4°
Dihydroquinine	B.2HBr, $3H_2O$	3·902	Water	−152·7	−153·2
Cinchonidine	B.2HBr, $2H_2O$	4·2575	Water	−112·5	−111·4
Dihydrocinchonidine	B.2HBr, $2H_2O$	2·0828	Water	− 91·9	− 90·6
Quinidine	B.2HBr, $3H_2O$	3·6163	Water	+209·5 to +210·7	+219·2
Dihydroquinidine	B.2HBr, $3H_2O$	3·6628†	Water +HBr	+196	+197·5
Cinchonine	B.2HBr	4·2355	Water	+163·2	+166·6
Dihydrocinchonine	B.2HBr	5·0233	Water	+142	+143·1

* Emde gives $[\alpha]_D^{25°}$ − 183·6° for *c* 3·9363.

† Average of 7 determinations: *c* = 3·6491 to 3·6836 and $[\alpha]_D^{22 \text{ to } 27°}$ = 193·2 to 197·6°.

It will be seen that the two sets of figures are in good agreement so far as cinchonidine and the four dihydro-bases are concerned, the differences ranging from 0·5 to 1·5, which are not greater than might be expected from two workers, making observations on the same substance. With quinine the difference amounts to 3·36°. Emde purified his quinine, first as the acid sulphate and finally as the dihydrobromide and there can be no doubt as to its purity. It is suggested that his lower specific rotation may be due to incomplete drying. Thus, he describes quinine dihydrobromide as a dihydrate, but the present authors found that the loss on drying at 120° *in vacuo* in seven samples varied from 10·12 to 10·86 % (average 10·37 %). The loss calculated for a trihydrate is 10·0 % and for a dihydrate 6·9 %.

In the case of cinchonine the difference is 3·4°. In this instance the salt is anhydrous and the higher figure now recorded is doubtless due to the complete elimination of dihydrocinchonine. Assuming this, the lower figure recorded by Emde corresponds to a cinchonine containing 14·3 % of dihydrocinchonine, which is close to the figure 15·1 % now found (p. 437) for commercial cinchonine after recrystallisation from alcohol until free from quinidine.

The figures for quinidine show the largest difference 8·5 to 9·7°. Emde found quinidine dihydrobromide to be a dihydrate whereas the present authors found

it to be a trihydrate. It is possible that in this case also Emde's lower figures are in part due to incomplete drying. Assuming that Emde really used a monohydrate instead of the anhydrous salt for his determination, his figure would be raised to $+217{\cdot}9°$, which on the basis of the present authors' results would correspond to quinidine containing 6 % of dihydroquinidine. This calculation assumes that the change in rotation due to dilution is the same and remains constant for quinidine and dihydroquinidine dihydrobromides. This is approximately true so far as the dilutions from $M/10$ to $M/40$ given in Table III are concerned, the two figures being 3·7 and 3·9°. The same assumption is made in the calculation for cinchonine given above and in this case the difference due to dilution of the dihydrobromides from $M/10$ to $M/40$ is 2·6° for both cinchonine and dihydrocinchonine.

Two further comparisons are possible with the results recorded in Table III. The Dutch Pharmacopoeia permits a range of $-17{\cdot}7°$ to $-18°$ for the observed rotation of $M/10$ solutions of quinine and its salts taken under specified conditions as to solvent and temperature. Under these conditions $M/10$ solutions of the quinine salts described in Table III gave the following results: monohydrochloride $-18{\cdot}06°$; neutral sulphate $-18{\cdot}095°$ and acid sulphate $-18{\cdot}085°$.

Realising the lack of any method of eliminating the large quantity of dihydroquinidine present in ordinary quinidine, a successful attempt was made recently [Butler and Cretcher, 1933] to prepare pure quinidine by re-arrangement of quinine [Rabe *et al.*, 1932]. The yield was apparently small. The product had $[\alpha]_D + 323{\cdot}8°$ ($c = 2$ in 1·8 % HCl). The pure quinidine used in the present investigation had $[\alpha]_D + 323{\cdot}1°$ observed under the same conditions.

The specific rotations, calculated as base in Tables III and IV, bring out the interesting point that all eight alkaloids in the form of dihydrobromides show a lower specific rotation in $M/10$ than in $M/40$ solution in water. It has not been possible to extend this comparison to other acid salts of all eight bases owing to the difficulty of getting satisfactory preparations of the complete series; the acid sulphates, for example, are too soluble to yield good preparations in some instances unless large quantities of the pure bases are available, and in other cases they cannot be dried completely without decomposition. It is however of interest to note that whilst quinine acid sulphate, like the dihydrobromide, shows a higher rotation at $M/40$ than at $M/10$, cinchonidine acid sulphate shows the same specific rotation at both concentrations. Determinations of p_H of these solutions, for which the authors are indebted to Mr C. G. Pope, throw no light on these differences. Anomalies of this kind in connection with the optical rotation of cinchona alkaloids have long been recorded [Oudemans, 1876] and have frequently been investigated, but so far no satisfactory and final explanation of their occurrence has been given.

Preparation of the drugs.

Separation of the primary alkaloids. It is easy to detect mere traces of quinine in cinchonidine, or of quinidine in cinchonine by the fluorescence of solutions in dilute sulphuric acid; and in purifying cinchonidine and cinchonine, each base was crystallised until a 1 % solution in $N/10$ sulphuric acid examined in bright, diffused daylight in a 2 dm. polarimeter-tube, showed no greater intensity of fluorescence than a 1 in 1,000,000 solution of quinine or quinidine respectively, examined in like manner. Under these conditions the fluorescence of quinine is just visible at 1 in 2,000,000.

The inverse operation, *viz.* the detection of cinchonidine in quinine has been much investigated, and in practice there are two methods in use, *viz.* the

ammonia test, which probably does not detect less than 2 % of cinchonidine, and the polarimetric test, which is of no value for the present purpose, since quinine is contaminated not only with cinchonidine, but also with the optically active bases, dihydroquinine and dihydrocinchonidine. Cinchonidine is less soluble in benzene than quinine, whilst its acid salts are more soluble in water than those of quinine: there is no reason to doubt that by rejection of appropriate fractions in fractional crystallisation from such solvents the small amount of cinchonidine in commercial quinine is eliminated.

The detection of cinchonine in quinidine is also difficult; there is a considerable difference between the optical rotations of the two alkaloids, but there has been until now no method of distinguishing between cinchonine and dihydroquinidine as the cause of low rotation in commercial quinidine. Cinchonine is less soluble in alcohol than quinidine and, it is shown later (p. 434) that it can be eliminated from crude quinidine by recrystallisation from boiling alcohol, the first fractions being rejected and any traces that escape in this process being left in the mother-liquors in the final fractionation, cinchonine dihydrochloride being much more soluble in water than quinidine dihydrochloride.

Elimination of dihydro-bases. The second group of impurities consists of the naturally-occurring dihydro-alkaloids, each primary alkaloid being accompanied by its own reduction product. The vinyl group in each of the parent bases is readily oxidised to a carboxyl group by permanganate, while the reduced bases are relatively stable to this reagent, and this method has hitherto been used both for the isolation of these dihydro-bases and for their estimation. Since the dihydro-bases are only relatively stable to permanganate, such a method can merely give approximate results and for the detection and estimation of the dihydro-bases, the authors have used a new method, *viz.* catalytic hydrogenation in an apparatus designed for quantitative work [Paget and Solomon, 1932]. The errors in such a determination are estimated not to exceed ± 1 %, and a primary cinchona alkaloid has been regarded as free from its dihydro-base when it absorbed 99·5 to 100·5 % of the quantity of hydrogen required to convert the vinyl group into an ethyl group. Colloidal palladium on barium sulphate or Adams's platinic oxide has been used as a catalyst, the former being preferred as it is less liable to form colloidal solutions from which the reduction product is difficult to recover. For the estimations, 0·4 to 0·5 g. of the base or one of its salts dissolved in 10 ml. of $N/10$ sulphuric acid, containing 0·05 g. of palladised barium sulphate, is exposed to hydrogen with constant, vigorous mechanical agitation. Absorption is rapid during the first half hour and finishes in about 2 hours after which it is taken as complete if, during a further 2 hours, the change in volume of gas is not greater than 0·1 ml. If, after this point is reached, action is continued, there is a slow absorption of 0·2 to 0·5 ml. *per diem* due no doubt to the very slow formation of more highly hydrogenated bases, but there is no practical difficulty in determining the point at which rapid formation of the dihydro-base is complete and the second very slow absorption begins. The results for hydrogen absorption given below are corrected for hydrogen absorbed by the catalyst, which must be determined by blank experiments on each new batch of catalyst made.

The bulk samples of dihydro-bases used were made by catalytic hydrogenation in like manner of the purified primary alkaloids and were crystallised as bases or salts to constant optical rotation. As so much attention has been given to the permanganate test for the dihydro-bases, it was thought desirable to use this in a more or less quantitative form. For that purpose, 5 ml. of a 2 % solution of the dihydro-base in water containing 0·3 ml. of N sulphuric acid was

treated with 0·5 ml. of $N/50$ potassium permanganate. The colour disappears in not less than 10 minutes with all the dihydro-bases except dihydrocinchonine, which discharges it in 30 seconds. The specific rotations of the pure bases and their salts are recorded in Tables III and IV. It is well known that in this series the specific rotation varies with the nature of the solvent and the concentration of the solution. As the authors had to make extensive use of polarimetric observations to follow the course of crystallisation in the final purification of the alkaloids, they found it convenient to use $M/40$ solutions of any salt selected for this purpose, since the series of readings were then directly comparable without calculation. The figures recorded for $[\alpha]_D$ in Tables III and IV are in all cases for $M/40$ or $M/10$ solutions of the dry salt or dry base in water or 0·1 N acid, usually 0·1 N sulphuric acid.

Additive compounds of cinchona alkaloids with metallic salts. The elimination of the dihydro-bases from quinine and cinchonidine is easy, and from cinchonine and quinidine slow and laborious. In the hope of improving it numerous salts and derivatives were tried. Quinidine sulphate argentonitrate, $B_2.H_2SO_4, AgNO_3$, proved useless, because it could not be recrystallised without decomposition. The zincichlorides of the type $B.(HCl)_2, ZnCl_2$, can be recrystallised, are easily purified, and the alkaloids are readily recoverable from them, but they are formed with equal facility and are of similar solubility with both the parent and the reduced bases and are therefore of no value as a means of separation. When this work was almost completed, a paper was published [Cohen, 1933] describing the specific precipitation by cupric chloride of cinchona alkaloids containing the vinyl group, and making the statement that "no complex salts are precipitated when the corresponding dihydro-bases are treated with cupric chloride under exactly the same conditions, even after several weeks at −4°." As the cuprichlorides are represented by the formula $B.(HCl)_2, CuCl_2$, which is strictly analogous with that found for the zincichlorides, it seemed curious that this specificity should be exhibited by cupric chloride and not by zinc chloride. The behaviour of cupric chloride with commercial cinchonine and commercial quinidine was investigated carefully and it was found that the cuprichlorides did, in fact, effect a partial separation of the dihydro-bases. The resulting fractionation showed clearly that the dihydro-bases must form insoluble cuprichlorides and, on trying the experiment, no difficulty was experienced in preparing cuprichlorides of all four dihydro-bases, except in the case of dihydrocinchonidine, where the additive compound is readily soluble in conc. HCl at atmospheric temperatures and when prepared in small quantities is best manipulated in a cold room.

In the following account, the melting-points quoted are corrected; the combustion results are for material dried at 120° *in vacuo* unless otherwise stated and were obtained by micro-analysis.

Quinine. The material used consisted of base prepared from commercial quinine sulphate. It had $[\alpha]_D^{15°}$ −280·7° ($c = M/40$ in $N/10$ H_2SO_4) and hydrogen absorption 94·7 %. It is known that cinchonidine can be eliminated from quinine by repeated crystallisation of the acid sulphate [Tutin, 1909]. The authors have found that quinine purified in this way is also free from dihydroquinine, as indicated by its absorptive capacity for hydrogen. Purification can also be effected by crystallisation of the dihydrobromide [Emde, 1932]. The quinine acid sulphate was prepared by repeated crystallisation (five or six times is usually necessary) from hot water (1 in 7 to 8) until the rotation became constant. It was then recrystallised in three fractions, for which $[\alpha]_D^{15°}$ showed the narrow range −216·1° to −217·0° ($c = M/40$ in water); the hydrogen absorption, for which the middle fraction was used, was 100·1 %. The dihydrobromide was prepared by recrystallisation from hot (43°) water (1 in 3 to 4) with the same precautions as the acid sulphate: the range of specific rotation for the three final fractions was −189° to −189·0°, the hydrogen

absorptions of the middle fractions were 100·8, 101·5 and 101·6 % (hydrogen absorptions by quinine dihydrobromide were generally high). The neutral hydrochloride and sulphate, made by neutralising pure quinine base with the appropriate acid, were recrystallised from hot water containing a trace of the appropriate acid to avoid any risk of inclusion of free base, which accounts for the high rotation shown by some specimens of neutral quinine salts.

Constants for the pure base and salts are given in Table III.

Dihydroquinine. The base, prepared by hydrogenation of pure quinine was crystallised from hot benzene, or as the neutral sulphate or the dihydrobromide from hot water. Constants for these three preparations are given in Table III.

Dihydroquinine cuprichloride. Dihydroquinine (5 g.) was dissolved in conc. HCl (8 ml.) and cupric chloride (5 g.) in conc. HCl (8 ml.) added: the mixture was kept at 7° for 24 hours, but nothing separated. The side of the beaker just under the surface of the liquid was then rubbed vigorously with a glass rod for 5 minutes. A yellow streak appeared and in 20 minutes the liquid had set to a semi-solid, crystalline magma. The crystals were filtered out on Whatman filter-paper No. 54, washed sparingly with conc. HCl and dried in a vacuous desiccator over soda lime; wt. 7·0 g. The product was recrystallised from 14 ml. hot conc. HCl; wt. 3·9 g. It crystallised on the sides of the container in silky, cushion-like masses of golden-brown needles, M.P. 204° (decomp.). (Found: C, 44·61; H, 5·22; N, 5·26; Cl, 26·60; Cu, 11·68 %. $C_{20}H_{26}O_2N_2.2HCl, CuCl_2$ requires: C, 44·98; H, 5·28; N, 5·25; Cl, 26·57; Cu, 11·9 %.)

Dihydroquinine zincichloride. $C_{20}H_{26}O_2N_2.2HCl, ZnCl_2$. The base (5 g.) dissolved in conc. HCl (8 ml.) was mixed with zinc chloride (12·5 ml. of 40 % solution in conc. HCl). Crystallisation began almost immediately, and the product was isolated in the same way as the cuprichloride. Yield 6·9 g. It crystallises from hot water (1 in 5) but shows a tendency to separate as an oil, which can be corrected by the addition of a little alcohol; also from hot 50 % alcohol or from hot conc. HCl in masses of colourless, flattened prisms, M.P. 257°; froths 262°. (Found: C, 45·12; H, 5·43; N, 5·28; Cl, 26·35; Zn, 12·20 %. Calc.: C, 44·82; H, 5·27; N, 5·23; Cl, 26·48; Zn, 12·22 %.)

Quinidine. The quinidine base used had $[\alpha]_D^{15°} + 324·3°$ ($c = M/40$ in $N/10$ H_2SO_4) and hydrogen absorption 77·9 % indicating the presence of 22·1 % of reduced bases, chiefly dihydroquinidine. After recrystallisation from boiling alcohol, the first fraction (about 10 %) being rejected, it had $[\alpha]_D^{15°} + 325·5°$ and hydrogen absorption 76·4 %; these slight changes are accounted for by removal of cinchonine. The hydrogen absorption could not be raised by crystallisation of either the base from alcohol, or the dihydrobromide from water, but the dihydrochloride (1 in 1 in hot water) proved suitable for this purpose, the less soluble fractions being richest in quinidine, as indicated by increasing rotation and hydrogen absorption. Three such crystallisations give a yield of about 33 % by weight of the starting material, and raise the hydrogen absorption to 90 %. This procedure answers well until a product having hydrogen absorption 98·0 to 98·5 % is reached. At this stage crystallisation from weaker solutions (1 in 3) ensures more rapid purification. At the first point at which approximate purity is reached, the yields are about 2·75 % of material with hydrogen absorption 100·6 % and about 7·5 % showing hydrogen absorption from 98 to 99 %.

In the course of attempts to find a derivative of quinidine, which would expedite the tedious fractional crystallisation necessary especially in the last stages of purification by means of the dihydrochloride, addition compounds of the alkaloid with silver nitrate and with zinc chloride were examined.

Quinidine argentonitrates. Two silver nitrate addition products of quinidine are known, one represented by the formula $(C_{20}H_{24}O_2N_2)_2, AgNO_3$ [Stenhouse, 1864], which cannot be recrystallised without decomposition, and a second, obtained as a crystalline precipitate when silver nitrate is added to dilute solutions of quinidine sulphate. Though used as a test for quinidine, the origin of this latter reaction has not been traced, and the nature of the precipitate appears to be unknown. It was prepared by adding silver nitrate solution (5 ml. of 50 %) to a boiling, aqueous, faintly acid solution of quinidine sulphate (5 g.). As the solution cooled, colourless needles of quinidine sulphate argentonitrate separated. Yield 6·2 g. The substance is sparingly soluble in boiling water, but can be crystallised with some decomposition from boiling 50 % alcohol in colourless needles containing $2H_2O$. (Loss at 120° *in vacuo* 3·9: calc. 3·7 %.) M.P. 213° (decomp.). (Found: C, 51·3; H, 5·98; N, 7·72; MeO, 6·32; Ag, 11·74; S, 3·51 %. $(C_{20}H_{24}O_2N_2)_2.H_2SO_4, AgNO_3$ requires: C, 52·3; H, 5·4; N, 7·6; MeO, 6·7; Ag, 11·7; S, 3·49 %.) The carbon determinations were per-

sistently low in recrystallised specimens, owing to the slight decomposition observed on recrystallisation, but there can be little doubt that the substance is correctly represented by the formula given. The "quinidine" recovered from the silver nitrate compound absorbed 81·1 % of the calculated amount of hydrogen, which is only a slight improvement on the original figure of 78 %.

Quinidine zincichloride. This was made by the usual method (p. 434) using quinidine 1 in 5 and zinc chloride 1 in 3 of conc. HCl. It crystallises from hot water or 50 % alcohol but best from hot conc. HCl (1 in 3) when it forms much-twinned colourless prisms, M.P. 242° (decomp.). In the following analyses the two figures for each constituent are for material crystallised from conc. HCl and water respectively and dried at 120° *in vacuo*. (Found: C, 44·98, 45·0; H, 5·13, 5·10; N, 5·32, 5·49; MeO, 6·02, 6·23; Cl, 25·9, 25·48; Zn, 12·5, 12·4 %. $C_{20}H_{24}O_2N_2.2HCl, ZnCl_2$ requires: C, 44·9; H, 4·87; N, 5·25; MeO, 5·8; Cl, 26·5; Zn, 12·2 %.) This is the formula adopted by Stenhouse for what was undoubtedly the same substance and the foregoing confirms and extends his description.

Quinidine cuprichloride. In view of the claim [Cohen, 1933] that the vinyl-containing cinchona alkaloids form solid cuprichlorides whilst the dihydro-bases do not, this method was tried on quinidine. The "quinidine" was not recovered by Cohen's method of removing copper as the sulphide but by the simpler process of dissolving and thereby decomposing the cuprichloride in cold water and pouring the filtered solution into excess of ammonia solution, when the base is precipitated and is readily filtered and washed free from the ammoniacal copper solution. The following is a typical result of a close imitation on a larger scale of one of Cohen's experiments with the modification just referred to, starting with a "quinidine" fraction having a hydrogen absorption of 51·6 %: Cohen used a mixture, quinidine-dihydroquinidine (1:1).

The "quinidine" (11 g.) was dissolved in conc. HCl (40 ml.) and cupric chloride (11 g.) in 36 ml. hot conc. HCl added. On cooling, the orange-coloured crystalline cuprichloride separated and was collected, dried overnight on a porous tile and finally in a vacuous desiccator over soda lime for 48 hours. Yield 11 g. A filtered aqueous solution of the cuprichloride (3 g. in water 30 ml.) was run into solution of ammonia (10 ml. conc. NH_4OH with water 30 ml.) and the precipitated base collected, thoroughly washed and dried in a vacuous desiccator. Yield 1·97 g. It absorbed 67·5 % of the calculated amount of hydrogen, *i.e.* an improvement of 15·9 %. In other trials with varying amounts of hydrochloric acid as solvent such results as the following were obtained; the figures are the hydrogen absorption values % of (*a*) the "quinidine" used, and (*b*) the recovered "quinidine."

(*a*)	77·9	77·9	86·6	78·6	80·4	88·1	95·8
(*b*)	91·0	88·0	88·7	80·4	88·1	95·8	98·0

The method has obvious possibilities and by combining it with the present authors' method—recrystallisation of the dihydrochloride—it is possible to obtain the latter in a pure state. The remainder of the "quinidine" cuprichloride (8 g.) prepared from the 51·6 % "quinidine" referred to above, was recrystallised from hot conc. HCl (1 g. in 3 ml.) and the whole of the crop (4·6 g.) ground in a mortar with water (9 ml.). Under these conditions the cuprichloride decomposed, and part of the quinidine dihydrochloride formed crystallised out. This was collected and without washing or drying was converted into base as already described. Yield 2·15 g. This absorbed 85·2 % of the calculated amount of hydrogen, an improvement of nearly 34 %. Still better quinidine but in poorer yield can be obtained by the use of more water (3 or 4 ml. instead of 2 ml. for 1 g. of cuprichloride). The base recovered from the dihydrochloride mother-liquors is of poorer quality.

Starting with "quinidine" showing hydrogen absorption 77·9 % and using conc. HCl (5 ml.) for each g. of quinidine or cupric chloride employed, it is possible by one treatment by this modified process, to obtain 1/3 of the original amount as a fraction showing hydrogen absorption about 95·4 % and by repeating the operation to obtain about 1/9 of the original amount having hydrogen absorption 99 to 100 %.

The quinidine used in determining the constants of the base, acid sulphate and dihydrobromide recorded in Table III was made by both processes. No figures are given for quinidine dihydrochloride as this substance is not completely dehydrated at 120° *in vacuo*, and between 120° and 130° the loss is irregular owing to incipient decomposition.

Dihydroquinidine. This base, prepared by catalytic hydrogenation of pure quinidine was crystallised from boiling alcohol, or as the dihydrobromide from water.

Dihydroquinidine cuprichloride was prepared by mixing the base (2 g.) with cupric chloride (2 g.) each having been dissolved separately in conc. HCl (5 ml.). Nothing separated on standing for 24 hours except a few crystals of cupric chloride, but on rubbing the side of the beaker, yellow granules appeared and the whole liquid quickly became semi-solid. Wt. 1·71 g. It was recrystallised from hot conc. HCl (1 in 1·5 ml.) from which it separated in clusters of thin, dark orange, rectangular prisms, M.P. 222° (decomp.). Yield 0·9 g. (Found: C, 44·94; H, 5·37; N, 5·17; Cl, 26·55; Cu, 11·92 %. $C_{20}H_{26}O_2N_2.2HCl, CuCl_2$ requires: C, 44·98; H, 5·28; N, 5·25; Cl, 26·57; Cu, 11·91 %.)

Dihydroquinidine zincichloride was prepared by mixing the base (2 g.) in conc. HCl (5 ml.) with zinc chloride (5 ml. of 40 % solution in conc. HCl). Nothing crystallised out until the inside of the beaker was rubbed vigorously with a glass rod. Wt. 1·7 g. It can be recrystallised from water, 50 % alcohol or hot conc. HCl (1 g. in 2 ml.) and separates from the latter in colourless, spheroidal masses of minute silky needles, M.P. 249–252° (decomp.). (Found: C, 44·6; H, 5·31; N, 5·19; Cl, 26·45; Zn, 12·00 %. $C_{20}H_{26}O_2N_2.2HCl, ZnCl_2$ requires: C, 44·82; H, 5·27; N, 5·23; Cl, 26·48; Zn, 12·22 %.)

Constants for dihydroquinidine and its salts are given in Table III.

Table III. *Constants of pure cinchona alkaloids.*

Methoxy-bases: quinine, dihydroquinine; quinidine, dihydroquinidine.

Substance	α_D	c*	Solvent	$[\alpha]_D^{15°}$ calc. for Salt	$[\alpha]_D^{15°}$ calc. for Base	Additional constants
Quinine (Q):						
Base, M.P. 173·5°	− 4·611°	*M*/40	*N*/10 H_2SO_4	—	−284·5°	H.A.† =99·93
Acid sulphate, $Q.H_2SO_4, 7H_2O$	−17·875	*M*/10	Water	−211·7°	−275·9	H.A. =100·1: p_H =2·86
,, ,,	− 4·555	*M*/40	,,	−216·1	−281·4	p_H =3·08
,, ,,	− 4·607	*M*/40	*N*/10 H_2SO_4	−218·3	−284·3	—
Neutral sulphate, $Q_2.H_2SO_4, 7H_2O$	− 4·608	*M*/40	*N*/10 H_2SO_4	−247·1	−284·4	—
Dihydrobromide, $Q.2HBr, 3H_2O$	−17·890	*M*/10	Water	−184·1	−276·2	H.A. =100·8: p_H =2·61
	− 4·608	*M*/40	,,	−189·6	−284·4	p_H =2·94
Neutral hydrochloride, $Q.HCl, 2H_2O$	− 4·597	*M*/40	*N*/10 H_2SO_4	−255·1	−283·8	—
	− 4·582	*M*/40	*N*/10 HCl	−254·3	−282·9	—
Dihydroquinine (HQ):						
Base, M.P. 173·5°	− 3·842	*M*/40	*N*/10 H_2SO_4	—	−235·7	—
Dihydrobromide, $HQ.2HBr, 3H_2O$	−14·880	*M*/10	Water	−152·5	−228·3	—
	− 3·845	*M*/40	,,	−157·5	−235·8	—
Neutral sulphate, $(HQ)_2.H_2SO_4, 6H_2O$	− 3·838	*M*/40	*N*/10 H_2SO_4	−204·6	−235·4	—
Quinidine (Qd):						
Base, M.P. 173·5°	+ 5·415	*M*/40	*N*/10 H_2SO_4	—	+334·2	H.A. =99·8 %
Acid sulphate, $Qd.H_2SO_4, 4H_2O$	+20·915	*M*/10	Water	+247·8	+322·7	—
	+ 5·409	*M*/40	*N*/10 H_2SO_4	+256·4	+333·9	—
Dihydrobromide, $Qd.2HBr, 3H_2O$	+21·315	*M*/10	Water	+219·3	+328·9	—
	+ 5·420	*M*/40	,,	+223·0	+334·5	—
Dihydroquinidine (HQd):						
Base, M.P. 169·5°	+ 4·875	*M*/40	*N*/10 H_2SO_4	—	+299	—
Dihydrobromide, $HQd.2HBr, 3H_2O$	+19·178	*M*/10	Water	+196·5	+294·2	—
	+ 4·890	*M*/40	,,	+200·4	+300	—

* c is expressed as dry substance.
† H.A. =hydrogen absorption: % of calculated value (p. 432).

Cinchonidine. Commercial cinchonidine is stated to contain quinine 10 % and dihydrocinchonidine 8 % [Dawson and Garbade, 1930]. The base, used as a starting material, showed strong fluorescence on solution in dilute sulphuric acid, had $[\alpha]_D^{15°}$ − 183·2° (c = *M*/40 in *N*/10 H_2SO_4) hydrogen absorption 97·9 % and contained methoxyl 0·66 %. These results indicate the presence of quinine (about 6·9 %) and dihydrocinchonidine (about 2 %).

It was found that crystallisation three times from boiling benzene (1 in 30), the first fractions only being collected, yielded about 78 % of a product, which passed the fluorescence test (p. 431) and in which no methoxyl could be detected. This, on fractional crystallisation from alcohol

(1 in 6), yielded first and third fractions having hydrogen absorptions of 98 to 99 % and a middle fraction with hydrogen absorption 101·2 %, the yield of the latter being about 25 % of the original material. In some cases it was necessary to crystallise the quinine-free product twice from alcohol, in order to reach this degree of purity. The constants for the base, acid sulphate and dihydrobromide are recorded in Table IV.

Dihydrocinchonidine. The base prepared by hydrogenation of pure cinchonidine was recrystallised from boiling benzene and finally from alcohol, or as the dihydrobromide from water. The constants are given in Table IV. The *cuprichloride* was prepared by mixing the base (3 g.) in conc. HCl (4 ml.) with cupric chloride (3 g.) in conc. HCl (5 ml.). Even after vigorous rubbing of the cooled liquid with a glass rod no crystals appeared, but after 24 hours at 7°, a crop of minute pale, canary-yellow needles separated, wt. 2·6 g. It was recrystallised in the same way from warm conc. HCl (3 ml.), wt. 1·5 g., and was dried on a porous tile. It sinters at 120–140°, melts at 166° and froths at 183°. (Found on substance dried at 90° *in vacuo*: C, 44·52; H, 5·29; N, 5·5; Cl, 28·15; Cu, 12·83 %. $C_{19}H_{24}ON_2.2HCl$, $CuCl_2$ requires: C, 45·27; H, 5·20; N, 5·56; Cl, 28·16; Cu, 12·62 %.)

Cinchonine. The commercial cinchonine used was markedly fluorescent in dilute sulphuric acid, contained methoxyl 0·25 %, corresponding to quinidine 2·6 % and had $[\alpha]_D^{17°}$ +264·5° ($c = M/40$ in $N/10$ H_2SO_4) and hydrogen absorption 86·2 % indicating the presence of 13·8 % of dihydrocinchonine. After three crystallisations from boiling alcohol (1 in 30) it passed the fluorescence test (p. 431) and the figure for hydrogen absorption fell to 84·9 owing to removal of quinidine. This partially purified base was converted into dihydrobromide and recrystallised from water (1 in 1). The first crop, which is rich in dihydrocinchonine is rejected and successive crops are obtained by slow concentration of the mother-liquor in a vacuous desiccator over sulphuric acid. When a conveniently large crop has accumulated in this way, it is scraped down into the mother-liquor, dissolved by warming and left to crystallise out again. The fourth crop so obtained had hydrogen absorption 93–94 %. When a hydrogen absorption about 95 % is reached, crystallisation is continued from stronger solutions (1:0·75). When material having hydrogen absorption over 99 % is first reached, the yields of the three best fractions are approximately as follows:

Hydrogen absorption, %	96 to 97	99·3	99·7
Yield, % of starting material	22·7	4·1	2·4

Purification of cinchonine by crystallisation of the dihydrobromide is slow and tedious and, in the hope of expediting the work, other derivatives were tried including the silver nitrate and the zinc chloride compounds, but neither of these was found effective as a means of eliminating dihydrocinchonine.

Cinchonine sulphate argentonitrate was prepared by adding silver nitrate solution (1 ml. of 50 %) to cinchonine sulphate (1 g. in 26 ml. water). On standing overnight a crop of colourless needles separated; wt. 0·984 g. This, on boiling with water (15 ml.), showed slight decomposition, but on filtration through charcoal gave a clear, faintly brown solution, which on cooling deposited nearly colourless needles (0·4 g.) containing $2H_2O$. (Loss at 130° *in vacuo* 4·26: calc. 4·27 %.) M.P. 199° (decomp.). (Found: C, 51·38; H, 6·01; N, 7·82; Ag, 12·88; S, 3·87 %. $(C_{19}H_{22}ON_2)_2.H_2SO_4$, $AgNO_3$ requires: C, 53·2; H, 5·41; N, 8·18; Ag, 12·5; S, 3·74 %.) As in the case of quinidine sulphate argentonitrate (p. 434), the carbon determinations were persistently low in the recrystallised material, owing no doubt to the slight decomposition already referred to.

Cinchonine zincichlorides. When a zinc chloride solution (4 ml. of 50 % in water) is added to cinchonine sulphate (1 g. in 26 ml. water) an oil is precipitated, which dissolves on addition of alcohol (10 ml.). On standing, a crop (0·9 g.) of colourless needles separates, which from solution in boiling water (20 ml.) deposits on cooling a crop of cinchonine hydrochloride. The mother-liquor on concentration in a vacuous desiccator deposits a *cinchonine hydrochloride zincichloride* represented by the formula $C_{19}H_{22}ON_2.HCl$, $ZnCl_2$. It can be recrystallised from boiling water or 50 % alcohol. M.P. 282° (decomp.). (Found: C, 48·69; H, 5·13; N, 6·39; Cl, 22·48; Zn, 14·4 %. Calc.: C, 48·8; H, 4·98; N, 6·0; Cl, 22·7; Zn, 14·0 %.) On solution in conc. HCl this substance is converted into a *zincichloride* of the usual type, $C_{19}H_{22}ON_2.2HCl$, $ZnCl_2$, which can also be formed in the usual manner by adding zinc chloride (2 g. in 3 ml. conc. HCl) to cinchonine (2 g.) in conc. HCl (10 ml.). This crystallises in anhydrous colourless, hair-like needles, from water, dilute alcohol

or conc. HCl (1 g. in 8 ml.). M.P. 263° (decomp.). (Found: C, 45·5; H, 5·08; N, 5·76; Cl, 28·04; Zn, 12·7 %. Calc.: C, 45·2; H, 4·80; N, 5·56; Cl, 28·1; Zn, 12·9 %.)

Two cinchonine zincichlorides have been described already [Gräzinghoff, 1865] to which the following formulae were assigned $(C_{19}H_{22}ON_2.2HCl)_2, ZnCl_2, 2H_2O$ and $(C_{19}H_{22}ON_2.3HCl)_2, ZnCl_2, H_2O$. It is suggested that these products were probably mixtures in which each of the two zincichlorides referred to above was still contaminated with cinchonine hydrochloride, which in the present instance separated as a first fraction in recrystallising the crude product.

The base recovered from the normal zincichloride made from crude cinchonine showed hydrogen absorption 84·0 % as against 86·2 % for the starting material, so that this derivative is of no value for purification purposes.

Cinchonine cuprichloride. The possibility of using the cuprichloride as a means of purifying cinchonine was investigated as described under quinidine (p. 435). After numerous trials, the best results were obtained by using equal quantities of cinchonine and cupric chloride dissolved separately to produce a final solution of solids (1 g.) in conc. HCl (8 ml.), recrystallising the resulting cuprichloride from hot conc. HCl (1 g. in 3 ml.), and recovering the base in the usual way from the recrystallised cuprichloride. Cinchonine dihydrochloride is very soluble in water so that no further fractionation can be effected by solution of the cuprichloride in a small quantity of water as in the case of quinidine (p. 435). The following are typical results:

	Hydrogen absorption, %			
Initial cinchonine	71·5	85·2	96·4	96 to 97
Recovered cinchonine	89·6	96·4	99·0	100·1
Yield, %	78·0	88·0	61·0	59·4

That is, starting with quinidine-free "cinchonine" showing hydrogen absorption of about 85 %, two passages through the cuprichloride are necessary to raise the hydrogen absorption to 99–100 %.

The cinchonine used for the final determination of the constants recorded in Table IV was prepared partly by fractionation of the dihydrobromide and partly by the cuprichloride method, and no difference could be detected between the two products.

Table IV. *Constants of pure cinchona alkaloids.*

Methoxyl-free bases: cinchonidine, dihydrocinchonidine; cinchonine, dihydrocinchonine.

Substance	α_D	c*	Solvent	$[\alpha]_D^{15°}$ calc. for Salt	$[\alpha]_D^{15°}$ calc. for Base	Additional constants
Cinchonidine (Cd):						
Base, M.P. 204·5°	− 2·617°	*M*/40	*N*/10 H_2SO_4	—	−178°	H.A.† = 101·2
Acid sulphate, $Cd.H_2SO_4, 5H_2O$	−10·498	*M*/10	Water	−133·9°	−178·5	H.A. = 100·2: p_H = 2·84
,, ,,	− 2·613	*M*/40	,,	−133·6	−178·1	p_H = 3·05
,, ,,	− 2·615	*M*/40	*N*/10 H_2SO_4	−133·4	−177·8	—
Dihydrobromide, $Cd.2HBr, 2H_2O$	−10·112	*M*/10	Water	−110·9	−172·0	p_H = 2·52
	− 2·607	*M*/40	,,	−114·3	−177·3	p_H = 2·89
	− 2·604	*M*/40	*N*/10 H_2SO_4	−114·2	−177·2	—
Dihydrocinchonidine (HCd):						
Base, M.P. 232°	− 2·140	*M*/40	*N*/10 H_2SO_4	—	−144·6	—
Acid sulphate, $HCd.H_2SO_4, 5H_2O$	− 8·417	*M*/10	Water	−106·8	−142·2	—
,, ,,	− 2·132	*M*/40	*N*/10 H_2SO_4	−108·3	−144·1	—
Dihydrobromide, $HCd.2HBr, 2H_2O$	− 8·149	*M*/10	Water	− 89·0	−137·7	—
,, ,,	− 2·128	*M*/40	,,	− 92·9	−143·7	—
Cinchonine (Cn):						
Base, M.P. 254°	+ 3·876	*M*/40	*N*/10 H_2SO_4	—	+263·7	H.A. = 100·1 %
Dihydrobromide, Cn.2HBr	+15·238	*M*/10	Water	+167	+259·2	H.A. = 99·74 %
	+ 3·867	*M*/40	,,	+169·6	+263·1	—
Dihydrocinchonine (HCn):						
Base, M.P. 267°	+ 3·342	*M*/40	*N*/10 H_2SO_4	—	+225·8	—
Dihydrobromide, HCn.2HBr	+13·154	*M*/10	Water	+143·6	+222·2	—
,, ,,	+ 3·347	*M*/40	,,	+146·2	+226·1	—

* c is expressed on dry substance.
† H.A. = hydrogen absorption: % of calculated value (p. 432).

Dihydrocinchonine. The base prepared by catalytic hydrogenation of pure cinchonine was finally purified by crystallisation from boiling alcohol, and from this the dihydrobromide was prepared in the usual way. Dihydrocinchonine differs from the three other dihydro-cinchona bases dealt with in this paper by decolorising permanganate in the standard test (p. 432) in 30 seconds. The *cuprichloride* was prepared by adding the base dissolved in conc. HCl (1 g. in 2 ml.) to cupric chloride in the same solvent (1 g. in 3 ml.). The yield was 0·9 g., which was reduced to 0·45 g. by recrystallisation from 1 ml. hot conc. HCl. From hot concentrated solutions it separates at once in golden-brown needles, but if crystallised slowly, it forms transparent much-twinned garnet-coloured prisms M.P. 212° (both forms). (Found: C, 45·20; H, 5·25; N, 5·54; Cl, 28·21; Cu, 12·31 %. $C_{19}H_{24}ON_2.2HCl$, $CuCl_2$ requires: C, 45·27; H, 5·16; N, 5·56; Cl, 28·16; Cu. 12·62 %.)

The constants found for the base and dihydrobromide are given in Table IV.

Biological tests[1].

Antimalarial activity. The comparison of the activity of the alkaloids was made with canaries infected with a strain of bird malaria kindly supplied by Sir Rickard Christophers. A mixture of the blood from infected birds diluted with 5–10 parts of saline, containing sodium citrate to prevent clotting, was injected under the pectoral muscles of a number of normal birds. The infected birds were bled by cutting off their heads and the feathers excluded by filtering the blood through glass wool. The blood and saline mixture was kept warmed at 37° during the period of the injections, as it was found that the latent period before the appearance of the parasites was considerably longer for the birds which were inoculated last, if this were not done. In the case of warmed blood the latent periods of the birds inoculated first and last do not differ by more than a day if all the injections can be completed in 15–25 minutes. To obviate any systematic error on this score the birds were arranged so that the average time of standing of the inoculum was constant for each group of birds used for the different drugs. The drugs were given orally by the method of Roehl through a thin piece of rubber tubing. The first dose was given 4 hours after the injection of the infecting material. Subsequent doses were given at daily intervals for the next 5 days, making 6 doses in all. The drugs were given in proportion to the weight of the bird, in the majority of cases, 5 or 2·5 mg. of base dissolved in 0·5 ml. per 20 g. weight of bird (most of the birds weighed 13–18 g.). The birds tolerate the material better if it is injected slowly. Six birds were usually used for each drug and the potency was estimated in comparison with that of quinine by taking the average time after infection at which parasites appeared in a particular group and comparing it with the average of the quinine group done at the same time. The authors consider this point of importance in the comparison of the activity of the drugs. The average time of appearance of parasites after inoculation and treatment with 6×5 mg. doses of quinine has varied from 16 to 23 days in these and other experiments not yet recorded. These differences show that unless in each batch of infected birds a group is treated with some drug to serve as a standard of curative activity, quantitative estimations of relative activity will suffer from a larger and almost incalculable error. The error of such comparisons has been very roughly calculated, and a preliminary estimate suggests that with two groups of six birds a difference in mean time of appearance of parasites of 1 day is significant and for two groups of three birds, a difference of 1·5 to 2 days.

Occasionally it has not been possible to detect parasites in treated birds for 30 days after the infection. These birds have been reinjected with infected blood

[1] G. A. H. Buttle and J. W. Trevan are responsible for all the experiments on animals.

and if they have shown parasites after the infection, they are considered to have been complete cures.

The results are shown in Tables C and D.

Table C is a typical group treated with quinine. It shows the differences obtained in a group of birds treated in the same manner.

Table D shows the results obtained in different experiments.

Table C.

Bird No.	Drug	Proportion of red cells containing parasites (days after inoculation) 17	18	19	20	21	22	25
45	Quinine				(Died 5th day)			
46	6 × 5 mg.	—	VVF	—	1/15	—	1/50	—
47		—	—	—	—	—	—	—
48		—	VVF	—	1/15	—	1/15	—
49		—	VVF	—	1/10	—	1/10	—
50		—	—	—	1/120	1/100	—	1/40

VVF indicates one or two parasites in several fields.

Table D.

Date	Control. Average time of appearance of parasites in untreated birds (in days)	Drug (6 doses in each case)	Dose mg. per 20 g. bird weight	Average time of appearance of parasites in treated birds (in days)
13. i. 31	7·0	Dihydroquinine	5	21·6 + 2 cures
		Quinine	5	18·2 + 1 cure
		Dihydroquinidine	5	15·6
		Cinchonidine	5	14·0
		Dihydrocinchonine	5	11·0
		Dihydrocinchonidine	5	9·3
2. ii. 31	5·9	*Quinine*	5	17·6
		Dihydroquinine	3·5	16·3
		Dihydroquinidine	5	16·0
		Cinchonidine	5	14·6
		Quinine	2·5	14·6
		Quinine	1	14·0
		Dihydrocinchonine	5	12·0 (1st dose 10 mg.)
		Dihydrocinchonidine	5	11·2 (1st dose 10 mg.)
18. iii. 31	4·0	*Quinine*	5	16·0
		Quinidine	5	14·0* (2 birds only)
		Cinchonine	5	12·3
7. iv. 31	5·6	*Quinine*	5	19·0* (1 bird only)
		Quinidine	5	17·0* (1 bird only)
		Cinchonine	5	13·0

* Six birds were used for each group, usually some died during the test; if there were less than three survivors, it has been noted in the last column.

Toxicity. The toxicity of certain of these substances on intravenous injection was examined by intravenous injection into mice. The relevant results are given in Table E.

The average lethal doses are recorded in the last column. The figures represent the estimate of the dose necessary to kill 50 % of the mice. The differences between the different alkaloids are probably not statistically significant, with the possible exception of dihydroquinidine.

Table E. *Results of toxicity tests by intravenous injection into mice.*

Drug	Dose in mg. of base per 20 g. mouse	Mortality	Estimate of average lethal dose of base for 20 g.mouse mg.
Quinine acid sulphate	1·2	0/10	1·9
	1·8	4/10	
	2·1	8/10	
	2·4	23/25	
Quinine dihydrochloride	1·05	1/10	1·4
	1·5	6/10	
	1·9	5/5	
	2·25	10/10	
Dihydroquinine acid sulphate	1·0	1/10	1·9
	1·25	0/10	
	1·5	0/10	
	2·0	11/20	
	2·5	5/5	
	3·0	5/5	
Quinidine dihydrochloride	1·0	0/10	1·6
	1·5	5/10	
	2·0	11/15	
Dihydroquinidine acid sulphate	1·0	6/15	1·3
	1·25	2/10	
	1·5	7/10	
Cinchonine acid sulphate	1·0	1/10	1·75
	1·5	2/10	
	2·0	7/10	
Dihydrocinchonine acid sulphate	1·26	0/10	2·0
	1·9	0/10	
	2·2	10/10	
Cinchonidine acid sulphate	1·5	0/10	1·85
	1·75	3/10	
	2·0	17/20	
Dihydrocinchonidine acid sulphate	1·9	0/10	2·0
	2·2	10/10	
Cinchonine chloride hydrochloride	1·3	0/10	1·55
	1·6	7/10	
	1·8	9/10	

REFERENCES.

Butler and Cretcher (1933). *J. Amer. Pharm. Assoc.* **22**, 414.
Cohen (1933). *J. Chem. Soc.* 996.
Dawson and Garbade (1930). *J. Amer. Med. Assoc.* **94**, 704.
Emde (1932). *Helv. Chim. Acta*, **15**, 357.
Giemsa (1932). Malaria Commission, League of Nations, CH/Malaria, 183.
—— and Oesterlin (1933). *Arch. Schiffs- Tropenhyg.* **37**, Beiheft No. 4.
—— Weise and Tropp (1926). *Arch. Schiffs- Tropenhyg.* **30**, 334.
Goodson, Henry and Macfie (1930). *Biochem. J.* **24**, 874.
Gräzinghoff (1865). *J. prakt. Chem.* **95**, 221.
Malaria Commission, League of Nations (1931). CH/Malaria, 158 and 167 (1).
—— (1933). Third General Report, p. 252.
Oudemans (1876). *Liebig's Ann.* **182**, 33.
Paget and Solomon (1932). *J. Chem. Soc.* 1198.
Rabe, Kolbe and Hochstätter (1932). *Liebig's Ann.* **492**, 242.
Sinton (1930). *Records of the Malaria Survey of India*, **1**, 465.
Stenhouse (1864). *Liebig's Ann.* **129**, 15.
Tutin (1909). *Pharm. J.* (iv), **29**, 600.
Van der Sleen and Drossart Lulofs (1931). Malaria Commission, League of Nations, CH/Malaria, 166.

LXII. A MACRO-METHOD FOR THE DETERMINATION OF IRON IN BIOLOGICAL MATERIAL BY A MODIFIED PERMANGANATE TITRATION.

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In the course of investigations on the metabolism of iron, it became necessary to determine relatively large amounts of iron in the presence of such interfering agents as organic matter and phosphate. It was found that the errors introduced by colorimetric micro-methods became enormously exaggerated when amounts of iron between 1 and 10 mg., or more, were determined. A survey of the existing methods which could be used for such amounts of iron showed many disadvantages.

Destruction of organic matter by ignition was extremely time-consuming; incineration with sulphuric acid was considered to present the advantages both of quickness and of removal of halogens which would interfere in subsequent steps in the procedure.

The gravimetric estimation of iron required either the removal of phosphate by molybdate, or the use of the basic acetate separation. These modifications, together with the general undesirability of using ammonia in the laboratory, ruled out this method of investigation.

The use of a sulphuric acid digestion made possible the volumetric estimation of iron. The permanganate method of Margueritte [Treadwell and Hall, 1924] was chosen because of the ease of determining the end-point and the fact that permanganate offers a self-contained indicator. The iron for this method must be in the ferrous condition. This was assured by reduction by nascent hydrogen in the presence of an excess of zinc and sulphuric acid.

The method has proved to be very satisfactory and extremely accurate within the limits of its application. It has been used in some 400 estimations of iron during the past year with uniform success. The results obtained by the method will be published elsewhere, the present paper giving the details of the modifications employed, the precautions and a few examples of its accuracy and possible applications.

Experimental.

Reagents.

Sulphuric acid. "Baker's Analysed," "C.P." brand, low in heavy metals.

Perchloric acid. Merck's brand 60 % perchloric acid, sp. gr. 1·54, low in heavy metals.

Zinc. "Baker's Analysed" zinc, "C.P." brand, 30 mesh granular, low in lead and iron.

Potassium permanganate N/*10 and* N/*100.* Standardised according to Treadwell and Hall [1924] against pure sodium oxalate.

Glassware. All glassware was of pyrex, extracted with 2 % HCl, chromic acid and rinsed in many changes of distilled water. The distilled water should be checked for the presence of iron and if necessary redistilled from treated glass vessels.

Destruction of organic matter.

(*a*) *Solutions of soluble iron compounds.* An amount of the iron solution containing 5–10 mg. of iron was measured into an 800 ml. Kjeldahl flask. 10 ml. of concentrated sulphuric acid and two glass beads were added and digestion commenced in a Kjeldahl digestion apparatus over a low flame. After charring was complete and a blood-red, apparently homogeneous solution was obtained, 0·5 ml. of perchloric acid was added. Digestion was then continued for one hour. Oxidation was invariably complete at this time and the solution was free from chlorides.

(*b*) *Faeces.* 1 g. samples were used when patients were being treated by some form of iron therapy. When a low iron content of the faeces necessitated the use of much larger samples than this, it was deemed advisable to use one of the many micro-methods. The faeces were prepared for analysis by careful drying, first on the water-bath and later in a desiccator. The sample should be kept as finely divided as possible during the drying process. The sample for analysis was further finely ground and sampled by quartering. 1 g. samples of this finely ground material were transferred directly to an 800 ml. Kjeldahl flask and completely washed into the flask by distilled water. 10 ml. of sulphuric acid and two glass beads were added. The digestion was allowed to proceed very slowly, the flasks being rotated from time to time until homogeneous mixtures, free from foam, were obtained. At this point the speed of digestion was increased until golden-red, translucent solutions resulted. 0·5 ml. of perchloric acid was then added and the digestion continued for a further hour.

(*c*) *Whole blood.* 2–10 ml. of whole blood were used for the analysis, depending on the degree of anaemia. The digestion was carried out in the same way as for faeces. (To facilitate digestion the Kjeldahl flasks containing the blood and sulphuric acid may be allowed to stand overnight before the digestion commences.)

(*d*) *Insoluble iron compounds.* Insoluble iron compounds were analysed in the same way as for faeces. The amount taken for analysis however should contain at least 5 mg. of iron as the metal.

Reduction.

The contents of the Kjeldahl flasks were diluted with 15 ml. of water and raised to the boiling-point with constant rotation. The sulphuric acid solution was then transferred quantitatively to 125 ml. Erlenmeyer flasks. 10 ml. of distilled water were added to the Kjeldahl flasks, boiled and transferred quantitatively to the Erlenmeyer flasks. The volume of transfer fluid should be kept as low as possible. Boiling out the Kjeldahl flasks was found to be absolutely necessary since, even with the greatest care, some etching of the glass occurs.

The contents of the Erlenmeyer flasks were allowed to cool completely, and 5 g. of granulated zinc were added. The flasks were then fitted with Bunsen valves and allowed to stand overnight.

When the reaction between the zinc and sulphuric acid was complete, a small sample of the contents of the flask was removed by means of a capillary tube and the fraction of a drop so removed tested for ferric iron with ammonium thiocyanate, using a porcelain spot plate.

Titration.

When reduction was complete the contents of the Erlenmeyer flasks were filtered quantitatively through No. 42 Whatman filter-papers. The flasks were washed by decantation and the final residue transferred by a stream of water to the filter-paper. The filtrate, including the washing, was collected in 400 ml. pyrex beakers. Washing and transferring were done in the cold as quickly as possible to avoid re-oxidation of the iron.

2 ml. of concentrated sulphuric acid were added to the contents of the beakers and titration with 0·1 *N* potassium permanganate carried out. During the titration the beakers were surrounded by a white background and the end-point taken as the first recognisable pink flush persisting for 30 seconds.

When the amount of iron was less than 10 mg. it was found advisable to use 0·01 *N* potassium permanganate. In this case all transfers from the beginning of the procedure should be made with the minimum amount of wash fluid. To diminish further the dilution of the iron, the final transfers and washings may be made with cold 1 % sulphuric acid. The addition of further sulphuric acid to the solution before titration may then be omitted. The final titration was done in white porcelain casseroles which facilitated identification of the end-point with certainty. It was found advisable to use a standard end-point at first, but when the eye became accustomed to the recognition of the end-point, this precaution was omitted. All titrations were done with a Folin micro-sugar burette of 5 ml. capacity, graduated in 0·02 ml. to facilitate splitting of drops.

Blank.

A blank was run on all reagents by carrying through the exact procedure without the addition of the iron-containing material. This blank was subtracted from the total titration figure.

Precautions.

The oxidation of organic matter and the removal of interfering agents by sulphuric acid digestion was found to be straightforward if the directions were closely followed. Time was saved by digesting as slowly as possible at first and only increasing the temperature after all danger of foaming was over.

Much time was spent in determining a satisfactory form of zinc to employ in reduction. The 30-mesh zinc suggested was found to be the right size to prevent floating, if added after the solutions to be reduced were cool. 5 g. of zinc are more than adequate for the usual amounts of iron handled and this amount of zinc will keep the iron in a reduced condition for 12 hours, if the Bunsen valves are made air-tight.

From the time the reducing flasks are opened, no delay should be made. Re-oxidation of the iron was found to be quite rapid; however, if the titration follows the transfer within 15 minutes, no appreciable change in the titre can be found. In order to reduce the time still further, at this point, filtration may be made through platinum cones if such are available.

Experimental results.

The method has been used for the analysis of iron in such compounds as ferric ammonium citrate, in the presence of organic matter, phosphates and chlorides. It has also been used in the estimation of iron in such biological

material as faeces and blood. The data presented in Tables I and II indicate the degree of duplication and accuracy which has been in general obtained by the method.

Table I. *Recovery of added iron from dried faeces.*

Weight of faeces g.	Iron content of faeces mg.	Iron added mg.	Total iron found mg.	Iron recovered mg.	Error mg.
1·000	9·90	9·98	19·95	10·05	+0·07
1·000	9·90	9·98	19·85	9·95	−0·03
1·000	9·90	9·98	19·85	9·95	−0·03
1·000	9·90	5·00	14·80	4·90	−0·10
1·000	9·90	5·00	14·83	4·93	−0·07

Table II. *Analyses of 1 % and 0·1 % ferric ammonium citrate solution containing iron-free organic matter*.*

ml. of solution	%	Iron present mg.	Iron found A mg.	Iron found B mg.	Iron found Average mg.	Error mg.
50	1	88·95†	88·95	88·95	88·95	±0·00
25	1	44·47	44·35	44·25	44·30	−0·17
10	1	17·79	18·15	18·05	18·10	+0·31
5	1	8·89	9·05	9·15	9·10	+0·20
50	0·1	8·89	8·60	8·55	8·57	−0·32
25	0·1	4·45	4·21	4·27	4·24	−0·21
10	0·1	1·78	1·70	1·71	1·705	−0·07
5	0·1	0·89	0·80	0·62	0·71	−0·18

* Filter-paper. † Calculated from repeated estimations of ferric ammonium citrate.

In Table I recovery of added amounts of a standard iron solution (ferric chloride prepared from pure iron wire) from faeces is given. For amounts of iron above 5 mg., recoveries to within 0·1 mg. were obtained. The duplication of results was good; variations between duplicates of 0·03 mg. of iron were found. The iron values for the solutions given were obtained by the customary gravimetric method.

Table II presents data obtained from the analysis of standard solutions of ferric ammonium citrate. This compound was used in the investigation for which this method was devised and represents iron attached to an organic radical. Further organic matter was added in the form of filter-paper during the digestion. The error in mg. varied from 0·07 to 0·32 throughout the range of dilutions. The amount of iron present varied from 0·89 to 88 mg.

Table III is included as an example of the use of the method for general biological analyses. The patients had varying degrees of anaemia. The haemoglobin value was determined in two cases by direct estimation from the oxygen capacity and in the third, by use of the Sahli haemoglobinometer standardised against the oxygen capacity. The volumes of blood taken varied from 2 to 10 ml.

Table III. *Determination of the iron content of haemoglobin.*

Patient	ml. blood	Iron mg./100 ml. blood	O_2-capacity vol./100 ml. blood	Hb g./100 ml. blood	% iron in Hb
1	6	53·2	19·95	14·82	0·359
2	10	38·2	—	11·39*	0·345
3	2	45·8	17·91	13·30	0·342

* Sahli.

The determined percentage of iron in haemoglobin found in all cases checked to within 0·02 %. The values found for the percentage of iron in haemoglobin were slightly higher than those calculated from the figures of Murphy *et al.* [1931], who used a micro-method, but in close agreement with those of Hufner [1894]. From the duplication of results it appears that the method lends itself very well to whole blood determinations.

Reports by Brock and Taylor [1934] show that the method may be satisfactorily used in dialysis and metabolic studies of iron, when this metal is present in gross amounts.

SUMMARY.

1. A detailed description is given of a modification of the permanganate titration method for the determination of iron.
2. The method is applicable to the determination of iron in the presence of organic material and phosphate.
3. Data are presented showing the degree of accuracy and duplication of the results.
4. The satisfactory use of the method in the determination of the percentage of iron in haemoglobin is demonstrated.
5. The method is recommended for the determination of iron in all biological material in which samples can be obtained containing more than 2 mg. of iron per g.
6. Within the limits specified for its application, iron may be determined within $\pm 0{\cdot}3$ mg.

One of us (J. F. B.) was Leverhulme Research Scholar of the Royal College of Physicians of London at the time that the work was done, and expresses his appreciation to the Science Committee of the College for the privilege of spending the year in Boston.

The expenses of the investigation were defrayed in part by a gift from the Smith, Kline and French Laboratories, Philadelphia, to the Harvard Medical School.

REFERENCES.

Brock and Taylor (1934). *Biochem. J.* **28**, 447.
Hufner (1894). *Arch. Anat. Physiol.* 130.
Murphy, Lynch and Howard (1931). *Arch. Int. Med.* **47**, 883.
Treadwell and Hall (1924). Analytical chemistry, vol. 2 (6th ed.), p. 99. (New York, John Wiley and Sons.)

LXIII. THE DIFFUSION OF SOLUBLE IRON COMPOUNDS *IN VITRO*. THE EFFECT OF ACIDS, BASES AND ELECTROLYTES.

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Although Blaud, when in 1831 he introduced his iron pill for the treatment of chlorosis, recognised the need for administering iron in large doses, this principle was largely overlooked until recent years, when it has been revived in the treatment of hypochromic anaemia. It is recognised to-day that adequate oral iron therapy requires considerably larger quantities than were employed ten years ago.

It has always been appreciated, nevertheless, that much of the iron administered by mouth is unabsorbed and excreted in the faeces. Recently it has been shown by Heath *et al.* [1932] that in idiopathic hypochromic anaemia at low levels of haemoglobin, parenterally administered iron in the form of iron citrate is converted approximately quantitatively into haemoglobin. This observation resolves the problem of adequate oral iron therapy into a problem of the absorption of iron from the gastro-intestinal tract.

To raise the haemoglobin level of an adult by 2 % per day, which can readily be achieved in uncomplicated cases by adequate oral iron therapy, requires the absorption from the gastrointestinal tract of approximately 50 mg.[1] of metallic iron per day. The introduction of that amount of iron daily by injection of any existing iron compound intended for parenteral administration is, for the patient, always an uncomfortable and sometimes a dangerous procedure. It is obvious therefore that there is as yet no adequate substitute for the oral administration of iron. Very few disadvantages have been demonstrated in the oral administration of large doses of iron, and in every way this form of therapy is advisable in hypochromic anaemia.

A study of absorption from the gastro-intestinal tract cannot be made, of course, *in vitro*, but there are certain physicochemical relationships which may play a part in gastro-intestinal absorption which can be so studied, *e.g.* the chemical reactions which will lead to the presence of iron in a soluble form in the gastro-intestinal tract and the effect of such conditions as the hydrogen ion concentration on these reactions and on the rate of dialysis of iron.

The object of the present investigation was to determine which forms of soluble iron most readily dialyse through cellophane membranes and what conditions affect the rate of dialysis.

[1] Taking 5 litres as an average figure for adult blood volume, 15·6 g. per 100 ml. as 100 % haemoglobin and 0·34 % as the iron content of haemoglobin.

Methods.

Complete assembly of the apparatus used for the series of dialysis experiments is shown in Fig. 1. Seamless cellophane tubing[1] was found to be the most satisfactory medium for dialysis. The tubing was cut into suitable lengths and soaked for one hour in distilled water to remove the glycerol preservative. Each section of tubing was made into a sac by double tying and sealing with

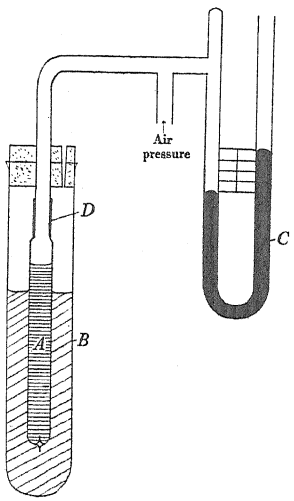

Fig. 1.

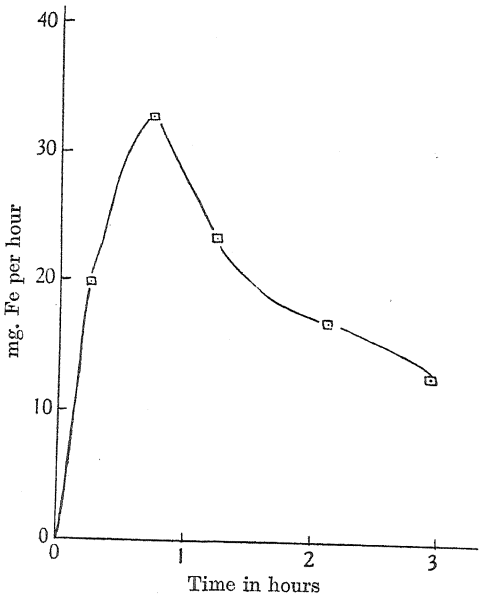

Fig. 2.

Fig. 1. Diagram of apparatus. *A*, cellophane sac containing iron mixture. *B*, diffusion medium. *C*, mercury manometer—20 mm. Hg. *D*, wax sealing cellophane on to glass tubing.

Fig. 2. Typical rate curve. Ferric ammonium citrate against serum.

collodion. Frequent checking throughout the study demonstrated that there was no leakage, and duplication of results was extraordinarily constant, varying by less than 4 % throughout the investigation. These cellophane sacs were sealed by means of paraffin wax, which made air-tight junctions, on to the ends of glass tubes projecting through rubber stoppers. The sacs were tested with applied pressure up to 40 mm. Hg. The iron solution to be studied was introduced into the sac and the diffusion medium into the outside container. Iron was subsequently determined in both the sac contents and the diffusion medium by means of a development of the Margueritte method for the volumetric determination of iron [Treadwell and Hall, 1924; Taylor and Brock, 1934]. By comparing the amount of iron recovered from the sac and from the diffusion medium at the end of the experiment with the amount introduced initially into the sac, it was possible to check accurately the entire procedure so that experimental errors were practically eliminated.

[1] Obtained from the Dupont Cellophane Company, 350, Fifth Avenue, New York, and having the following specifications: inflated diameter $\frac{3}{8}$ in., flat width 0·59 in., thickness 0·0006 in.

In order to find the optimum time for iron diffusion, the following experiment was devised. 4 ml. of a 10 % aqueous solution of ferric ammonium citrate were introduced into each of 5 sacs and diffused against normal horse-serum under an applied pressure of 20 mm. Hg. A sac was removed at the end of $\frac{1}{2}$, 1, $1\frac{1}{2}$, 3 and 12 hours. The rate of diffusion at each of these time intervals in mg. iron per hour was determined. These values were averaged for the time interval concerned and plotted against time. A typical curve is shown in Fig. 2. From this experiment it was observed that the curve of diffusion definitely flattens after 3 hours.

In all the experiments a constant positive pressure of 20 mm. Hg was applied to the inside of the sacs. Three different diffusion media were employed during the investigation, normal horse-serum, a special diffusion medium and distilled water. The special diffusion medium referred to in the text as "special saline," was introduced to avoid a protein precipitation which sometimes occurred with serum. It is essentially a protein-free blood-serum. The composition was based on the determination of the constituents of normal human serum as given by Mathews [1930]. 2 litres of solutions contained

Potassium sulphate	0·584 g.
Potassium chloride	0·746 ,,
Sodium chloride	11·520 ,,
Disodium hydrogen phosphate	0·564 ,,
Sodium bicarbonate	3·200 ,,
Calcium hydrogen phosphate	0·620 ,,
Magnesium chloride	0·464 ,,

The rate of diffusion was determined of a 10 % aqueous solution of ferric ammonium citrate into each of the three diffusion media.

Experimental.

First, the effect of various conditions on the rate of diffusion of ferric ammonium citrate was studied. Secondly observations were made on the rate of diffusion of some other soluble iron compounds, and the results were compared with those of ferric ammonium citrate. All experiments were performed in duplicate.

(1) *The effect of increasing concentration on the rate of diffusion of ferric ammonium citrate.*

5, 10 and 20 % aqueous solutions of ferric ammonium citrate were dialysed against normal horse-serum for the following periods: $\frac{1}{2}$, 1, $1\frac{1}{2}$ and 3 hours. In each case 4 ml. of the solution were introduced into the sac and 20 ml. of normal horse-serum into the container. The results are shown in Fig. 3, in which the percentage diffused was plotted against time. It will be observed that the three curves practically coincide. When these results are plotted in the form of an absolute rate curve, Fig. 4, it will be observed that the amount passing through the membrane in a given time is directly proportional to the concentration within the sac.

(2) *The effect of acid and alkali on the rate of diffusion of ferric ammonium citrate.*

10 % solutions of ferric ammonium citrate were made up in distilled water, 0·1 *N* HCl and 0·1 *N* NaOH. 4 ml. of each of these solutions were dialysed against 20 ml. of normal horse-serum for $\frac{1}{2}$, 1, $1\frac{1}{2}$ and 3 hours. The p_H of the

solutions varied from 1·5 in the case of the acid iron solution to 12·0 in the case of the alkaline iron solution. The results are given in Fig. 5.

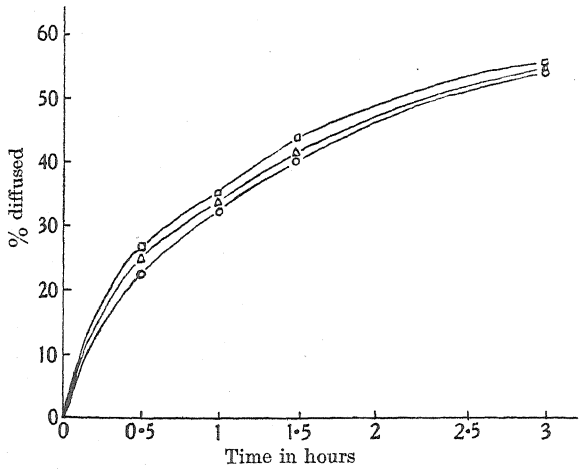

Fig. 3.

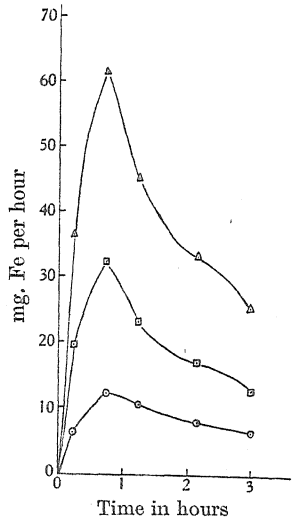

Fig. 4.

Fig. 3. Effect of concentration on the rate of diffusion of ferric ammonium citrate into serum. □ 20 %; △ 10 %; ○ 5 %.

Fig. 4. Rate curves. △ 20 % ferric ammonium citrate; □ 10 % ferric ammonium citrate; ○ 5 % ferric ammonium citrate.

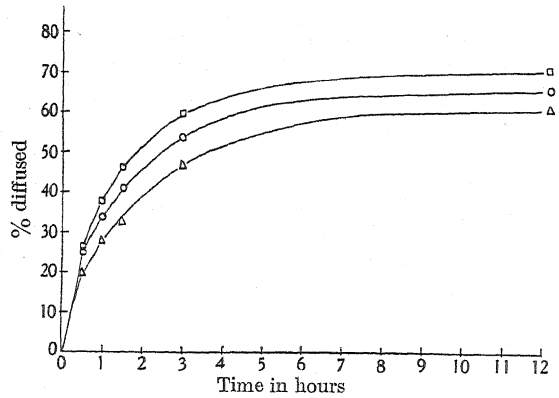

Fig. 5.

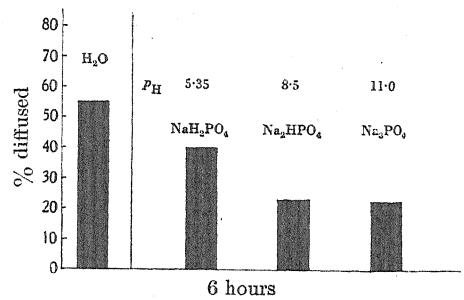

Fig. 6.

Fig. 5. Effect of acid and alkali on the diffusion rate of ferric ammonium citrate into serum. □ 0·1 *N* HCl; ○ H_2O; △ 0·1 *N* NaOH.

Fig. 6. Effect of 0·7 *M* phosphate ion on the diffusion of ferric ammonium citrate into serum.

The figure shows that the rate of diffusion from an acid medium is slightly faster than that from distilled water and the rate from an alkaline medium is slightly slower.

(3) *The effect of sodium phosphate on the rate of diffusion of ferric ammonium citrate.*

Sufficient Na_2HPO_4 was added to an aqueous solution of ferric ammonium citrate to make solutions containing 10 % ferric ammonium citrate which were respectively 0·035, 0·07, 0·35 and 0·7 *M* with respect to Na_2HPO_4. Similar solutions were made using NaH_2PO_4 and Na_3PO_4. 4 ml. of each of these 12 solutions were dialysed against 20 ml. of normal horse-serum for 6 hours. The results for the 0·7 *M* phosphate solutions are given in Fig. 6. In the case of NaH_2PO_4, the p_H of which was 5·35, there was a slight inhibition of the rate of dialysis as compared with a simple 10 % aqueous solution of the ferric ammonium citrate. In the case however of the two alkaline phosphates, the p_H values of which were 8·5 and 11·0, the inhibition of diffusion was very considerable. No precipitate whatsoever could be detected in any of these solutions. From a study of the other phosphate concentrations, it was found that the effect of the phosphate ion decreased with decreasing concentration. In the case of NaH_2PO_4, the inhibition of the rate of diffusion of the iron salt was of the same order as that found for neutral sodium chloride. For the alkaline phosphates, however, the depression of the rate of diffusion by the higher concentrations was approximately twice that of neutral sodium chloride.

The absolute rate of diffusion of ferric ammonium citrate in 0·7 *M* Na_2HPO_4 is plotted against time in Fig. 7. Special saline was used as the diffusion medium. It will be observed that there was almost complete inhibition of the diffusion of ferric ammonium citrate and that what diffusion did occur, proceeded at a constant rate. Since no precipitate was found during the experiment, it was necessary to consider the possibility that the iron and phosphate together were modifying the capillary permeability of the cellophane membrane by forming a true adsorption compound within the pores of the membrane. Such phenomena occur when copper sulphate and potassium ferrocyanide are independently diffused through porcelain membranes, resulting in a complete change in the character of the membrane. Such adsorption compounds are usually permanently fixed in the membrane. In order to test this possibility, 10 % aqueous solutions of ferric ammonium citrate were diffused for 1·5 hours into normal horse-serum. The sacs were then washed out thoroughly in distilled water, and a 10 % solution of ferric ammonium citrate in 0·7 *M* Na_2HPO_4 was diffused through the same membrane for 1·5 hours. The sac was once more thoroughly washed out in distilled water, and a 10 % solution of ferric ammonium citrate was diffused again through the membrane. The results are given in Fig. 8 and show that the rate of diffusion of the ferric ammonium citrate through the membrane was exactly the same after the passage of iron and phosphate together as it was before.

(4) *The effect of some other electrolytes on the diffusion of ferric ammonium citrate.*

The effect of some other electrolytes on the rate of diffusion of ferric ammonium citrate was studied. 10 % solutions of these substances—ammonium chloride, sodium citrate, sodium bicarbonate and sodium chloride—were used, in which ferric ammonium citrate had been dissolved to give also a concentration of 10 % with respect to the iron salt. 4 ml. of each of these solutions were then diffused against 20 ml. of special saline for $\frac{1}{2}$, 1, $1\frac{1}{2}$ and 3 hours. In this experiment special saline was preferred to serum in order to avoid the

occasional precipitation of serum-proteins by the electrolytes. The results are shown in Fig. 9, in comparison with a block for the diffusion of 10 % ferric ammonium citrate in distilled water under the same conditions. These various electrolytes decreased the rate of iron diffusion at the 3-hour period by amounts varying from 8 % for ammonium chloride and potassium citrate to 18 % for

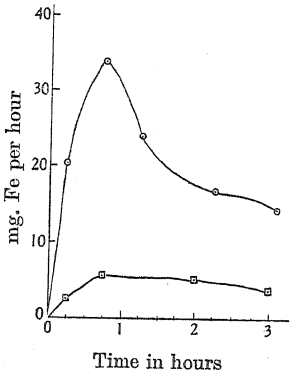

Fig. 7.

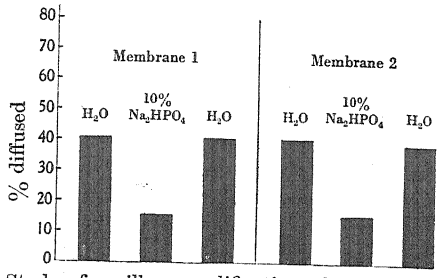

Fig. 8.

Fig. 7. Effect of phosphate. ○ Ferric ammonium citrate in H_2O; □ Ferric ammonium citrate in 0·7 *M* Na_2HPO_4.

Fig. 8. Showing the failure of ferric ammonium citrate in the presence of phosphate ion to change the characteristics of cellophane membranes.

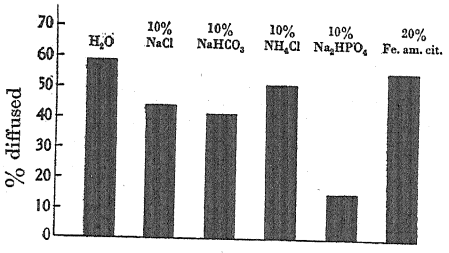

Fig. 9.

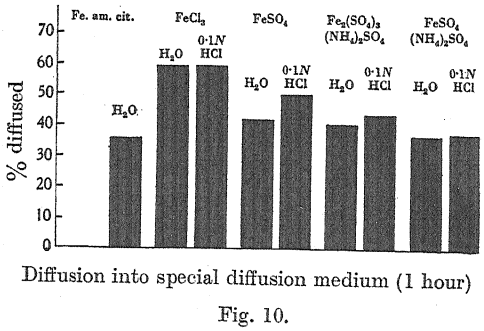

Fig. 10.

Fig. 9. Effect of various electrolytes on the diffusion of ferric ammonium citrate into special diffusion medium.

Fig. 10. Effect of acid on the diffusion of some iron salts.

sodium bicarbonate. There seems to be no relationship between the action of those electrolytes which are acid on hydrolysis and of those which react as bases. In spite of the common citrate ion, neither potassium nor sodium citrate decreased the rate of iron diffusion below that found for sodium chloride. Fig. 9 demonstrates clearly the striking inhibitory action of the alkaline phosphates on the diffusion of ferric ammonium citrate, while Fig. 6 demonstrates that the acid sodium phosphate has a much weaker inhibitory effect, which is of the same order as those of the other electrolytes tested.

(5) *A comparison between the diffusion of ferric ammonium citrate and that of some other soluble iron compounds.*

The rates of diffusion of ferric chloride, ferrous sulphate, ferrous ammonium sulphate and ferric ammonium sulphate were studied under conditions comparable with those used in the case of ferric ammonium citrate. 4 ml. of a 10 % solution of ferric ammonium citrate, which were used in that study, contained 70 mg. of metallic iron. Therefore, concentrations of the above salts were used such that 4 ml. contained 70 mg. of metallic iron. One- and three-hour periods were used in these experiments, and special saline was employed as a diffusion medium. Each of the iron salts was studied both in solution in distilled water and in 0·1 *N* HCl. The results are given in Fig. 10. The rate of diffusion of each of these salts was greater than that of ferric ammonium citrate, and with the exception of ferric chloride, the diffusion rate out of 0·1 *N* HCl was greater than out of distilled water. Ferric chloride, being an extremely acid salt, was naturally enough not affected by the presence of 0·1 *N* HCl.

All four of these soluble iron salts formed insoluble precipitates when they were added in the concentrations used in these experiments to 0·7 *M* Na_2HPO_4.

Discussion.

The data concerning the effect of increasing the concentration of ferric ammonium citrate on the diffusion of this salt need no comment. Two possible explanations can be offered for the effect of acid and alkali. The membrane itself may be modified by the presence of hydrogen or hydroxyl ions in contact with it, or the acid and alkali may interact directly with the ferric ammonium citrate to form substances having a higher or lower diffusion rate. The effect of electrolytes in general was to decrease the rate of diffusion of ferric ammonium citrate to a moderate degree. This observation is explicable on the ground that when electrolytes are present a portion of the osmotic work is being done by the electrolytes, and consequently, during any given time, a smaller number of molecules of ferric ammonium citrate pass through the membrane. Sodium dihydrogen phosphate acted in the same manner as the other electrolytes tested. The alkaline sodium phosphates however behaved quite differently. From the study of the true rate curve of iron diffusion (Fig. 7), it appears that after reaching a very low rate of diffusion the rate continues at a constant level. It was thought at first that adsorption compounds were formed within the membrane so that instead of a cellophane membrane being present, a cellophane membrane impregnated with ferric phosphate resulted. The crucial experiment described above, indicated quite clearly that such was not the case. In the absence of a precipitate the only reasonable explanation that could be offered was that the membrane became loosely plugged with a colloidal ferric phosphate. The fact that the presence of salts having a common citrate ion did not decrease the rate of diffusion below that found for sodium chloride may be considered evidence that ferric ammonium citrate diffuses through cellophane membranes largely as an undissociated molecule. From this study it has appeared that ferric ammonium citrate, which was chosen because it is a freely soluble iron compound and is used widely as a therapeutic agent, is in some ways unique. It is the only one of the five soluble iron compounds studied, which does not form a precipitate in 0·7 *M* Na_2HPO_4. Secondly, it is the only soluble iron salt which is extensively used as a therapeutic agent. The other forms of therapeutic iron in common use for oral administration being ferrum reductum and ferrous carbonate in the form of Blaud's pill.

It is of interest to compare with the above observations, certain results of investigations *in vivo* on the response of the organism to certain forms of orally administered iron. Mettier and Minot [1931] have shown that in hypochromic anaemia the reticulocyte response following oral administration of ferric ammonium citrate is greater when the iron is administered in an acid-buffered medium than when it is given in an alkaline-buffered medium. It was shown by Marlow and Taylor [1934] that the urinary excretion of iron is raised when ferric ammonium citrate is given orally in acid-buffered solutions, but not when it is given in alkaline-buffered solutions. These two observations indicate that ferric ammonium citrate, at any rate, is more readily absorbed when it is administered in an acid-buffered solution. Mettier [1930] found that the acid-buffered beef-steak medium, which he used, very materially altered the p_H of the duodenum for 2–3 hours, the time being longer when achlorhydria was present. The fact that the acid-buffered medium had a more prolonged effect in increasing the hydrogen-ion concentration of the duodenum of achlorhydric individuals than in the case of patients with a normal gastric acidity is interesting, although Mettier makes no comment on it. It appears to indicate that achlorhydric individuals are also deficient in their power to secrete alkali into the duodenum. Macallum [1893] and Abderhalden [1900] have claimed that iron is principally absorbed from the duodenum. Mitchell and Miller [1929] have shown that the ash of spinach, which contains iron, copper and manganese, is a better haemoglobin-building supplement to a milk diet in anaemic rats when the ash is dissolved in hydrochloric acid than when it is mixed in insoluble form with the food paste. It was shown by Cox *et al.* [1931] that soluble aluminium salts and ferric salts, when added to the diet of guinea-pigs in excess of the phosphorus present, cause a marked lowering of the inorganic phosphorus of the blood and of the calcium and phosphorus in the ash. This effect can be prevented by the addition to the diet of sodium dihydrogen phosphate in amount equivalent to the iron or aluminium. They state that these effects are due to the precipitation of alimentary phosphorus as the ferric and aluminium phosphates.

Brock and Diamond [1934], working with rats, have shown that the addition of large amounts of ferric or ferrous salts to a non-rachitogenic diet produces rickets. They have shown that the rickets can be prevented by the addition to this iron-containing diet of sufficient phosphate theoretically to combine with all the iron present. This work was undertaken as a sequel to the observations described in this paper on the effect of phosphates on the dialysis of iron and ammonium citrate.

So little is known about absorption from the gastro-intestinal tract that it is impossible to do more than speculate on the interpretation of some of these observations. But they do indicate the probability that reactions in the gastro-intestinal tract between iron and acids, bases and electrolytes may play an important rôle in determining the availability of that iron for absorption by the organism. Studies *in vitro* of such reactions may therefore lead to knowledge of the conditions favouring the absorption of iron.

Summary.

1. The rate of dialysis of certain soluble iron compounds across cellophane membranes has been studied under various conditions.

2. The rate of dialysis of ferric ammonium citrate is directly proportional to the concentration of the salt.

3. The rate of dialysis of ferric ammonium citrate into serum and into a special non-protein diffusion medium is increased by the presence of 0·1 *N* HCl. It is decreased slightly by 0·1 *N* NaOH, moderately by the presence of a variety of electrolytes, including sodium dihydrogen phosphate, and very markedly by secondary and tertiary sodium phosphates, in the absence of precipitates.

4. The rates of dialysis of certain other soluble iron compounds are compared with that of ferric ammonium citrate.

5. Certain observations *in vivo* recorded in the literature on the response of man and animals to various forms of iron therapy are set out for comparison.

The authors express their appreciation to Dr George R. Minot for his interest and co-operation during the progress of this investigation and to Miss Eleanor R. Shea for painstaking technical assistance.

One of us (J. F. B.) was Leverhulme Research Scholar of the Royal College of Physicians of London during the investigation and expresses his thanks to the Science Committee of the College for the privilege of spending the year in Boston.

The expenses of the investigation were defrayed in part by a gift from the Smith, Kline and French Laboratories, Philadelphia, to the Harvard Medical School.

REFERENCES.

Abderhalden (1900). *Z. Biol.* **39**, 113.
Brock and Diamond (1934). *J. Pediat.* (in press).
Cox, Dodds, Wigman and Murphy (1931). *J. Biol. Chem.* **92**, *Proc.*, xi.
Heath, Strauss and Castle (1932). *J. Clin. Invest.* **11**, 1293.
Macallum (1893). *J. Physiol.* **16**, 268.
Marlow and Taylor (1934). *Arch. Int. Med.* (in press).
Mathews (1930). Physiological chemistry (5th ed.), p. 479. (New York, William Wood and Co.)
Mettier (1930). *J. Clin. Invest.* **8**, 561.
—— and Minot (1931). *Amer. J. Med. Sci.* **171**, 25.
Mitchell and Miller (1929). *J. Biol. Chem.* **85**, 355.
Taylor and Brock (1934). *Biochem. J.* **28**, 442.
Treadwell and Hall (1924). Analytical chemistry, vol. **2** (6th ed.), p. 99. (New York, John Wiley and Sons.)

LXIV. A SIMPLE METHOD FOR THE PURIFICATION OF *CITRUS* PECTIN.

By HOMI RUTTONJI NANJI and JAMSHEDJI JIJIBHOY CHINOY.

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The preparation of highly purified pectin has hitherto depended upon the use of electrodialysis, prolonged settling of impurities [*cf.* Emmett, 1926] and similar methods. In any event, it is doubtful whether pure samples of esterified pectin have been obtained; only calcium pectate and pectic acid were regarded as obtainable in a pure state. The development of a method for preparing the esterified (soluble) pectin in a state of purity was, therefore, desirable. It has been found possible to do this readily by removing the impurities usually associated with pectins (prepared from *Citrus* fruits) by a method involving the use of iodine and bromine as oxidising agents in aqueous solution.

Norman and Norris [1930] had observed that pectin is very stable to oxidation with bromine in presence of calcium carbonate. Block and Cowgill [1932] had already used a method of oxidation to remove gums *etc.* from antineuritic vitamin extracts.

Iodine and bromine are usually employed as oxidising agents in alkaline solution. The presence of any alkali is evidently ruled out in this case on account of the extreme susceptibility of the ester groups in pectin to alkalis. However, it was found that oxidation of the impurities readily took place in a neutral or faintly acid medium without any appreciable effect on the pectin. Dilute solution of hydrogen peroxide was also tried without success.

Experimental.

Purification by partial oxidation with halogens.

General method. Pectin solution, about 0·5 %, was prepared by dissolving the pectin in boiling water. To 50 ml. of the cooled pectin solution about 3 ml. of $N/10$ iodine solution were added. The oxidation was allowed to proceed for varying periods and at different temperatures. The excess of iodine was then removed by shaking two or three times with ether until the aqueous layer was colourless. The excess could also be removed by warming *in vacuo* at 40°, or by bubbling air through the solution at the same temperature. Pectin was then precipitated by 95 % alcohol and after two hours filtered on muslin, finally passed through various grades of alcohol and dried in the oven at 100° for 20 hours.

Two commercial varieties of pectin were purified and a sample of grape fruit pectin, obtained by extraction of the rind with $N/75$ HCl, was similarly treated. In each series a control experiment was carried out, in which the pectin was precipitated without treating with iodine. The methoxyl value[1] and calcium pectate value[2] of the purified samples of pectin were determined (Table I).

[1] The MeO values were determined by the micro-Zeisel method [Pregl, 1930].

[2] The calcium pectate values were determined by taking an aliquot portion of the purified pectin dried at 100° for 20 hours, using the method of Carré and Haynes [1922].

Table I. *Purification with iodine.*

Sample of pectin	Time of oxidation	Temperature of oxidation ° C.	Yield of purified pectin g.	MeO %	Calcium pectate %
Series I:					
1. Commercial pectin I	Control	20	0·23	9·6, 9·6	94·9, 94·78
2. ,,	12 hours	20	0·21	10·6, 10·5	100·0, 100·15
3. ,,	20 ,,	20	0·20	11·0, 11·15	100·4, 100·4
4. ,,	48 ,,	20	0·19	11·4, 11·4	100·7, 100·8
5. ,,	72 ,,	20	0·19	11·6, 11·56	100·9, 100·8
6. ,,	½ hour	90–95	0·18	11·1, 11·0	100·5, 100·7
7. ,,	1 ,,	90–95	0·16	10·9, 10·8	100·2, 100·2
8. ,,	1 ,,	40	0·19	11·5, 11·4	100·8, 100·7
9. ,,	2 hours	40	0·18	11·4, 11·28	100·6, 100·38
10. ,,	6 ,,	40	—	10·9, 10·7	100·8, 100·7
Series II:					
1. Commercial pectin II	Control	20	0·22	7·9, 7·9	93·9, 94·0
2. ,,	24 hours	20	—	8·1, 8·2	96·0, 96·5
3. ,,	48 ,,	20	—	9·1, 9·2	100·0, 100·1
4. ,,	5 days	20	0·19	9·3, 9·2	100·4, 100·3
5. ,,	10 ,,	20	0·17	9·5, 9·4	100·5, 100·4
6. ,,	1 hour	40	0·20	9·4, 9·38	100·7, 100·6
7. ,,	2 hours	40	0·19	9·3, 9·3	100·3, 100·1
8. ,,	6 ,,	40	0·18	8·8, 8·5	100·3, 100·4
9. ,,	½ hour	90	0·18	9·1, 9·26	100·4, 100·3
10. ,,	4 hours	90	0·16	8·6, 8·5	100·2, 100·3
Series III*:					
1. Grape fruit† pectin	Control	20	0·22	9·4, 9·4	93·1, 93·2
2. ,,	1 day	20	0·20	11·2, 11·2	100·1, 100·2
3. ,,	2 days	20	0·20	11·3, 11·2	100·7, 100·5
4. ,,	6 ,,	20	0·18	11·3, 11·3	100·7, 100·8
5. ,,	1 hour	40	0·19	11·3, 11·38	100·4, 100·3
6. ,,	2 hours	40	0·17	11·6, 11·5	100·6, 100·6

* It will be recalled that Norman [1928], while attempting to prepare pure pectins from lemons and oranges, always obtained pectin having an apparent methoxyl value 8·5 %, although the real methoxyl content calculated from calcium pectate yield was 11·4. This low methoxyl value, he suggested, was due to admixture with partially de-esterified pectin and other degradation products produced by the action of the acid of the juice. This difficulty is readily overcome by using the rind instead of the entire fruit.

† Prepared by extraction of the grape fruit rind with *N*/75 HCl; MeO and Ca pectate values corrected for ash.

It will be seen that purification was slow at ordinary temperature, but at 40° it was complete in about 1–2 hours. Longer heating or heating at higher temperatures only tended to cause a slight hydrolysis of the methoxyl groups. The yield of oven-dried purified pectin in all cases was over 70 % of the starting material. Whilst the calcium pectate values showed a removal of 5–6 % of the "impurity," the methoxyl values were raised by 10–20 %. This would tend to show that halogens destroyed some pectin with lower methoxyl content. Also, in Series II, the highest methoxyl value obtained was 9·5 %; this was probably because the sample was a mixture of tri- and tetra-methoxypectic acid.

Purification with bromine water.

The purification was carried out exactly as described with iodine (p. 456) using approximately 20 ml. $N/10$ bromine water for each gram of pectin (Table II).

Table II. *Purification with bromine water.*

Sample of pectin	Time of oxidation	Temperature of oxidation ° C.	Yield of purified pectin g.	MeO %	Calcium pectate %
Series I:					
1. Commercial pectin I	Control	20	0·23	9·6, 9·6	94·9, 94·78
2. ,,	24 hours	20	0·21	11·2, 11·2	100·3, 100·4
3. ,,	48 ,,	20	0·20	11·5, 11·45	100·8, 100·9
4. ,,	72 ,,	20	0·20	11·5, 11·6	100·9, 100·9
5. ,,	1 hour	40	—*	11·4, 11·4	100·7, 100·6
6. ,,	2 hours	40	0·18	11·2, 11·2	100·6, 100·4
7. ,,	½ hour	90	—*	10·9, 10·8	100·3, 100·4
8. ,,	1 ,,	90	0·16	10·5, 10·4	100·2, 100·3
Series II:					
1. Commercial pectin II	Control	20	0·22	7·9, 7·9	93·9, 94·0
2. ,,	2 days	20	0·20	9·6, 9·58	100·1, 100·2
3. ,,	4 ,,	20	—*	9·6, 9·5	100·6, 100·8
4. ,,	1 hour	40	—*	9·6, 9·5	100·5, 100·5
5. ,,	2 hours	40	0·18	9·2, 9·1	100·3, 100·4
Series III:					
1. Grape fruit pectin	Control	20	0·22	9·4, 9·4	93·1, 93·2
2. ,,	24 hours	20	—*	11·3, 11·4	100·3, 100·4
3. ,,	48 ,,	20	0·20	11·3, 11·5	100·6, 100·8
4. ,,	1 hour	40	0·18	11·5, 11·4	100·3, 100·4
5. ,,	2 hours	40	0·18	11·5, 11·4	100·5, 100·5

* Not determined.

On comparing the above results with those obtained with iodine, it will be seen that bromine water was even more satisfactory than iodine, for its action was quicker; also, the excess of bromine was more readily removed, and the purified pectin was perfectly white.

Purification with chlorine water.

Attempts were made to carry out the purification by means of chlorine water under conditions similar to those described above, but without success. Partial de-esterification of the methoxyl groups and degradation of the pectin molecule itself were found to occur. Complete degradation occurred in 3 days at room temperature and in 1 hour at 40° with the production of brown gummy substances whose nature has not been ascertained.

Purification with hydrogen peroxide.

1 g. of the pectin dissolved in 200 ml. water was treated with 10 ml. of hydrogen peroxide (20 vols.) for varying periods at room temperature and the pectin was recovered as usual. At ordinary temperature no change in the methoxy land calcium pectate values was observed (this fact is in conformity with the observations of Norman and Norris [1930]). After treatment at higher temperatures, however, the pectin was completely oxidised, and no precipitate was obtained on addition of alcohol. The nature of these oxidation products is under investigation.

Action of halogens on sodium pectate and pectic acid.

As the halogens seemed to have slight action on partly de-esterified pectin it was of interest to study their action on other pectic substances such as sodium pectate and pectic acid.

(A) *Sodium pectate.* Preliminary experiments showed that the action of iodine on sodium pectate was very slow. Sodium pectate (2 g.) was dissolved in water and the solution divided into eight equal parts. Each part was treated with an excess (20 ml.) of $N/10$ iodine solution for varying periods at different temperatures, the excess of halogen was removed, and the sodium pectate was precipitated by alcohol (2 vols.), washed, dried at 100° for 20 hours and weighed (Table III).

Table III. *Action of iodine on sodium pectate.*

Time of treatment	Temperature (° C.)	Yield of sodium pectate recovered (g.)
Control	Room	0·24
24 hours	,,	0·24
72 ,,	,,	0·21
120 ,,	,,	0·20
240 ,,	,,	0·19
24 ,,	40–50*	0·22
72 ,,	40–50*	0·20
48 ,,	90*	0·20

* A fresh quantity of $N/10$ iodine solution was added when the solution became almost colourless.

Approximately 20 % of sodium pectate was destroyed in 10 days at ordinary temperatures, in 3 days at 40–50° and in 2 days at 90°.

(B) *Pectic acid.* 0·2 g. samples of pectic acid were weighed in a flask, suspended in water and treated with 20 ml. of $N/10$ bromine or iodine solution as shown in Table IV. (More halogen was added from time to time to replace the loss at higher temperatures.) Alcohol (2 vols.) was then added to precipitate any pectic acid dispersed in solution; the precipitate was collected, washed with alcohol, dried at 100° for 20 hours and weighed (Table IV).

Table IV. *Action of halogen on pectic acid.*

	Time of treatment	Temperature ° C.	Yield of pectic acid recovered g.
1. Control	—	—	0·20
2. Treatment with iodine	2 days	Room	0·20
3. ,,	3 ,,	,,	0·19
4. ,,	10 ,,	,,	0·19
5. ,,	3 ,,	50	0·18
6. ,,	6 ,,	50	0·17
7. ,,	3 ,,	90	0·16
8. Treatment with bromine	3 ,,	50	0·17
9. ,,	5 ,,	50	0·16

The action of halogen on pectic acid is negligible at room temperature and slight even at higher temperatures; this is probably due to its insolubility.

Action of halogens on the impurity isolated from a commercial Citrus *pectin.*

The "impurity," probably hemicellulose, from commercial pectin was obtained by allowing the pectin solution to stand overnight with sufficient lime water to render the solution just alkaline. After removing the precipitated calcium pectate, excess calcium was removed by adding oxalic acid till just

neutral, filtering and precipitating the filtrate with alcohol. 20 g. *Citrus* pectin gave 0·75 g. of the "impurity," *i.e.* 3·75 %; the latter contained about 23 % inorganic matter.

This isolated impurity (0·9 g.) was dissolved in water, the solution divided into three equal parts and then treated with excess of iodine or bromine solution at 40° for 6 hours. At the end of that period, alcohol (2 vols.) was added, and the insoluble impurity recovered was dried at 100° for 20 hours and weighed. As treatment with iodine failed to destroy about 33 % of the impurity the experiment was repeated, increasing the time of reaction to 12 hours. It was extraordinary to find that about 30 % of the impurity was still unattacked. This was possibly due to some change, physical or chemical, in part of the impurity during isolation. That this was actually the case was proved by an indirect procedure. A sample of pectin was first treated with iodine as on p. 456, and an attempt to isolate the impurity, by the method described above, was made. The product isolated from 20 g. of purified pectin weighed about 0·1 g. and consisted entirely of inorganic matter.

The results of treatment of the impurity with halogens are recorded below.

	Yield of ash-free impurity recovered g.
1. Control	0·17
2. Treatment with iodine for 6 hours	0·08
3. Treatment with iodine for 12 hours	0·07
4. Treatment with bromine for 6 hours	0·01

Application of the above methods to the preparation of pure pectin from plant materials.

The method was used in the preparation of very pure samples of pectin from *Citrus* fruits (grape fruit and lemon). The white inner rind of these was used, and extraction was carried out by means of hot $N/75$ HCl. (Extraction with water gave very small yields, and 0·5 % ammonium oxalate removed an excessive amount of non-pectic substance.) The extraction was carried out by adding about 10 times the quantity of acid to the powdered rind, and after keeping at ordinary temperature overnight, boiling for 1–2 hours on a water-bath; the extract was then filtered off at the pump. The cooled pectin extract was then directly treated with a fair excess of $N/10$ bromine water or $N/10$ iodine solution (iodine is not as satisfactory as bromine, for reasons already stated), and kept for 2 days at room temperature. The excess of halogen was then removed by ether, the pectin precipitated with alcohol and treated in the usual manner.

In certain cases, the method detailed above gave a slightly coloured product, owing to the presence of certain impurities which gave red to violet colorations with iodine. Also in other cases impurities which readily absorb halogens were present, which necessitated the use of a large quantity of the oxidising agent. Both the above difficulties could, however, be avoided if the pectin was precipitated by alcohol immediately after extraction, redissolved in water and then treated with the oxidising agent.

The results of extraction and purification of grape fruit and lemon pectin are recorded in Table V.

Determinations of total furfuraldehyde and the uronic anhydride content of the several samples of pectin, after purification by the methods evolved, were

Table V.

Sample of pectin	% yield (calculated on wt. of air-dried rind)	MeO % (Mean of two determinations)	Ca pectate % (Mean of two determinations)	Remarks
Series I:				
1. Lemon pectin without purification	25	9·4	95	Deep yellow-coloured product
2. Lemon pectin directly treated with iodine	21·8	11·4	100·2	Slightly coloured product
3. Same as 2 with bromine	20·8	11·5	100·4	White product
4. Lemon pectin precipitated after extraction, redissolved and then treated with iodine	20·0	11·7	100·6	White product
5. Same as 4 with bromine	18·9	11·6	100·7	White product
Series II:				
1. Grape fruit pectin without purification	24·1	9·8	95·3	—
2. Grape fruit pectin directly treated with iodine after extraction	20·7	11·7	100·6	—
3. Same as 2 with bromine	20·0	11·6	100·7	White product
4. Grape fruit pectin precipitated after extraction, redissolved and then treated with iodine	18·0	11·7	100·8	White product
5. Same as 4 using bromine	17·2	11·7	100·8	White product

carried out in order to test the agreement between the values obtained and those required by the formula given to the basal molecule of pectic acid by Nanji, D. R. *et al.* [1925] (Table VI).

Table VI.

	Furfuraldehyde %		Uronic acid anhydride %	
	Found	Calculated*	Found	Calculated*
Commercial pectin I	20·28	—	69·60	—
,, II	20·40	—	69·80	—
Grape fruit pectin	20·38	20·45	69·80	69·60
Lemon pectin	20·31	—	69·70	—

* Based on Nanji, Paton and Ling's formulae.

Discussion.

The purification of pectin with bromine and iodine in aqueous solution gives a rapid method of preparing pectin in a state of extreme purity, both from fairly pure commercial samples of pectin and also from *Citrus* fruits. The method would be particularly useful for investigations involving the structure of pectin, for unless a perfectly pure uniform sample of pectin were available conclusions regarding its ultimate constitution would have to be accepted with reserve. It is intended to extend the method to pectins from sources other than *Citrus* fruits.

The furfuraldehyde and carbon dioxide figures for purified pectin, as well as the action of halogens on sodium pectate and pectic acid, clearly show that no violent change has been produced in the pectins themselves by the action of halogens. The stability of the methoxyl groups in pectin towards oxidising agents is remarkable. Some evidence is already at hand to show that the fully methoxylated pectic acid is more stable to oxidation than the partly de-esterified pectin.

A full discussion of the function of methoxyl groups in pectin and the part they play in the genesis of pectin must be reserved till the work now in hand is completed; but it may be suggested that their function appears to be to prevent biological oxidation of pectin in plants.

SUMMARY.

1. Samples of pectin prepared from *Citrus* fruits and containing 90–95 % pectin can be further purified by treatment with bromine water or iodine at temperatures between 20° and 40°.

2. Chlorine water under similar conditions causes breakdown of the pectin: dilute hydrogen peroxide is without action.

3. The furfuraldehyde and carbon dioxide yields from pectins purified by this method agree with those usually accepted for highly purified pectin.

4. The methoxyl content of certain samples is found to approach very nearly that required by a fully methoxylated pectic acid.

5. The halogens have little action on sodium pectate and pectic acid.

The authors wish to record their thanks to the Coleer Pectin Company, New York and the California Fruit Growers Exchange for supplying commercial samples of *Citrus* pectin.

REFERENCES.

Block and Cowgill (1932). *J. Biol. Chem.* **96**, 127.
Carré and Haynes (1922). *Biochem. J.* **16**, 60.
Emmett (1926). *Biochem. J.* **20**, 564.
Nanji, D. R., Paton and Ling (1925). *J. Soc. Chem. Ind.* **44**, 235 T.
Norman (1928). *Biochem. J.* **22**, 749.
—— and Norris (1930). *Biochem. J.* **24**, 402.
Pregl (1930). Quantitative organic microanalysis, p. 181. (Churchill.)

LXV. THE SUPPLEMENTARY RELATIONSHIPS BETWEEN THE PROTEINS OF WHEAT AND RYE BREADS AND THOSE OF THE PEA (*PISUM SATIVUM*).

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It has been reported by Kon and Markuze [1931] that a supplementary relation exists between the proteins of soya bean flour and those of wheat and rye flour. The present paper deals with the effect of an addition of pea meal (from *Pisum sativum*) to the two latter substances on the biological value of their proteins.

Experimental.

The biological values of the proteins of pea meal, rye bread (baked from 70 % rye flour by the Municipal Bakeries in Warsaw), of two mixtures of wheat rolls and pea meal and of two mixtures of rye bread and pea meal have been determined by the method of Osborne *et al.* [1919].

The bread was dried at 35° and the dry product ground in a mill. The peas were boiled, dried and ground. The following mixtures were used: wheat-pea mixture I; wheat rolls 80 %, pea meal 20 %; wheat-pea mixture II; wheat rolls 60 %, pea meal 40 %; rye-pea mixture I; rye bread 80 %, pea meal 20 %; rye-pea mixture II; rye bread 60 %, pea meal 40 %. The nitrogen contents of the above products are given in Table I. The composition of the diets is given in Table II. The biological values of the proteins were, in most cases, estimated at

Table I.

Product examined	Protein content (calculated on dry wt.) N × 6·25 (%)	Ratio of bread- to pea-proteins
Rye bread from 70 % rye flour	7·4	—
Pea meal	26·2	—
Wheat-pea mixture I	14·0	1·8 : 1
Wheat-pea mixture II	16·8	1 : 1·5
Rye-pea mixture I	11·2	1·1 : 1
Rye-pea mixture II	14·6	1 : 2·4

several levels of protein intake. Albino rats bred in the laboratory were used in the experiments. The technique of the tests was as described by Kon and Markuze [1931]. The results will be found in Table III.

It will be seen from the tables that for the pea-meal-proteins alone a biological value of from 1·2 to 1·4 was found at 3 levels of protein intake. On the other hand the values found for the wheat-pea mixture I were from 1·6 to 1·9, while

Table II.

All values refer to dry weights

Source of protein	Composition of diets			
	Level of protein intake (N × 6·25)			
		7·1 %		
Rye bread from 70% rye flour	Bread	95		
	Salts*	5		
	Level of protein intake (N × 6·25)			
		6·2 %	8·2 %	12·5 %
Pea meal	Pea meal	22·9	30·5	45·8
	Butter fat	10·0	10·0	10·0
	Salts	5·0	5·0	5·0
	Potato starch	62·1	54·5	39·2
	Level of protein intake (N × 6·25)			
		6·1 %	8·8 %	11·2 %
Wheat-pea mixture I	Mixture	42·8	57·1	80·0
	Butter fat	10·0	10·0	10·0
	Salts	5·0	5·0	5·0
	Potato starch	42·2	27·9	5·0
	Level of protein intake (N × 6·25)			
		6·3 %	8·1 %	11·0 %
Wheat-pea mixture II	Mixture	35·7	47·6	65·5
	Butter fat	10·0	10·0	10·0
	Salts	5·0	5·0	5·0
	Potato starch	49·3	37·4	19·5
	Level of protein intake (N × 6·25)			
		6·2 %	8·2 %	10·5 %
Rye-pea mixture I	Mixture	53·5	71·4	95·0
	Butter fat	10·0	10·0	—
	Salts	5·0	5·0	5·0
	Potato starch	31·5	13·6	—
	Level of protein intake (N × 6·25)			
		6·3 %	8·5 %	11·8 %
Rye-pea mixture II	Mixture	41·0	55·0	75·5
	Butter fat	10·0	10·0	10·0
	Salts	5·0	5·0	5·0
	Potato starch	44·0	30·0	9·5

* Steenbock and Nelson's [1923] salt mixture No. 40.

the second wheat-pea mixture gave at all three levels a value of at least 2·0, *i.e.* twice that found by Kon and Markuze [1931] for the proteins of wheat rolls. In all cases the biological value of the proteins of the mixtures exceeds that of the proteins of the separate constituents.

A similar supplementary relation was also found for the rye-pea mixtures. In the case of the rye-pea mixture I the biological value was 2·0 and 2·1 for the two lower levels and 1·6 for the highest level; it varied from 1·5 to 1·6 for all three levels of the second mixture. Rye bread alone gave a definitely lower value of 1·1. Owing to its low nitrogen content this bread could be fed only at one level of protein intake.

Results similar to those found in the case of wheat-pea mixtures have been observed when wheat gluten was fed to rats either as the sole source of protein or combined with pea meal. The mixture was composed of 80 % of pea meal and 20 % of wheat gluten (containing 84·2 % of protein). The two diets were fed

Table III.

Source of protein in diet	Protein (N × 6·25) content* %	Rat no.	Days	Total intake: Food* g.	Total intake: Protein* g. = *a*	Initial wt. g.	Gain in wt. g. = *b*	Biological value of protein *b*/*a*	Mean
Rye bread from 70% rye flour	7·1	827 ♀	31	236·8	16·8	66	14	0·83	1·14
		902 ♀	31	346·5	24·6	61	30	1·22	
		903 ♀	31	329·6	23·4	64	27	1·15	
		830 ♀	31	303·1	21·5	69	29	1·35	
Pea meal	6·2	786 ♀	31	183·1	11·3	46	14	1·24	1·21
		789 ♂	31	177·3	11·0	45	13	1·18	
	8·2	785 ♀	31	200·0	16·4	45	20	1·22	1·21
		788 ♂	31	223·3	18·3	49	22	1·20	
	12·5	784 ♀	31	242·0	30·2	40	41	1·36	1·38
		787 ♀	31	245·8	30·7	41	43	1·40	
Wheat-pea mixture I	6·1	745 ♀	31	302·5	18·4	55	35	1·90	1·92
		746 ♀	31	235·7	14·4	60	28	1·94	
	8·8	747 ♀	31	306·7	27·0	60	45	1·67	1·57
		749 ♂	31	285·8	25·1	58	37	1·47	
	11·2	748 ♀	31	349·8	39·2	63	66	1·68	1·77
		750 ♂	31	264·4	29·6	68	55	1·86	
Wheat-pea mixture II	6·3	752 ♀	31	285·9	18·0	54	39	2·17	2·11
		755 ♂	31	294·3	18·5	60	38	2·05	
	8·1	753 ♀	31	319·5	25·9	53	49	1·89	2·08
		754 ♀	30	330·8	26·8	53	61	2·27	
	11·0	740 ♀	31	330·8	36·4	59	80	2·20	2·00
		751 ♀	31	401·5	44·2	68	84	1·90	
Rye-pea mixture I	6·2	829 ♀	31	256·1	15·9	55	40	2·51	1·99
		833 ♂	31	250·3	15·5	75	25	1·61	
		881 ♀	31	304·8	18·9	49	35	1·85	
	8·2	831 ♀	31	311·2	25·5	62	55	2·16	2·07
		832 ♂	31	337·3	27·7	76	55	1·98	
	10·5	826 ♀	31	322·9	33·9	72	54	1·59	1·56
		837 ♂	31	299·2	31·4	56	48	1·53	
Rye-pea mixture II	6·3	840 ♀	31	288·5	18·2	56	30	1·65	1·56
		843 ♂	31	368·1	23·2	68	34	1·46	
	8·5	841 ♀	31	356·0	30·3	64	47	1·55	1·52
		844 ♂	31	355·0	30·2	71	45	1·49	
	11·8	842 ♀	31	341·8	40·3	74	56	1·39	1·47
		845 ♂	31	349·0	41·2	79	64	1·55	

* Calculated on dry weight.

Table IV.

Source of protein	Composition of diet* %		Protein (N × 6·25) content* %	Rat no.	Days	Total intake: Food* g.	Total intake: Protein* g. = *a*	Initial weight g.	Gain in weight g. = *b*	Biological value of protein *b*/*a*	Mean
Wheat gluten	Gluten	14·2	11·8	966 ♂	31	195·6	23·1	46	22	0·95	1·00
	Butter fat	10·0		967 ♂	31	187·2	22·1	43	20	0·90	
	Salts	5·0		968 ♂	31	244·0	29·0	47	33	1·14	
	Potato starch	70·8									
Gluten-pea mixture	Mixture	34·2	11·6	983 ♀	31	264·0	30·6	58	57	1·86	2·24
	Butter fat	10·0		984 ♀	31	294·2	34·1	57	78	2·29	
	Salts	5·0		985 ♂	31	285·0	33·1	56	85	2·57	
	Potato starch	50·8									

* Calculated on dry weight.

only at one level of protein intake; their composition and the results of the animal tests are given in Table IV. The biological value of the gluten proteins was found to be 1·0, which corresponds closely with the value obtained by Kon and Markuze [1931] for wheat roll proteins. The biological value of the proteins of the gluten-pea mixture is 2·2, *i.e.* about twice as great as in the case of wheat gluten alone and almost double that of the proteins of pea meal.

SUMMARY.

1. The biological values of the proteins of wheat gluten, of rye bread baked from 70 % rye flour, of pea meal and of mixtures in various proportions of pea meal with wheat rolls, rye bread and wheat gluten have been determined by the method of Osborne, Mendel and Ferry.

2. A supplementary relation exists between the proteins of wheat rolls and rye bread and those of pea meal.

3. The same is true in the case of a mixture of wheat gluten and pea meal.

REFERENCES.

Kon and Markuze (1931). *Biochem. J.* **25**, 1476.
Osborne, Mendel and Ferry (1919). *J. Biol. Chem.* **37**, 223.
Steenbock and Nelson (1923). *J. Biol. Chem.* **56**, 355.

LXVI. THE ABSORPTION OF *n*-HEXADECANE FROM THE ALIMENTARY TRACT OF THE CAT.

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(*Received February 28th, 1934.*)

In continuance of previous studies of factors affecting the nature and amount of the unsaponifiable fractions of liver, it was shown that *n*-hexadecane is absorbed from the alimentary tract of the rat to the extent of 50 to 108 mg. daily, depending on the quantity of hydrocarbon included in the diet [Mahdi and Channon, 1933]. This result is of interest in relation to fat absorption, because it is opposed to current ideas that absorption of paraffinoid substances does not occur. A point of further importance arose in the course of this work. In one experiment, of 149 g. of hexadecane administered, only 116 g. were excreted, and of the 33 g. of hexadecane unaccounted for no more than an insignificant trace, if any, was present in the liver, whilst the unsaponifiable fraction of the carcass contained at most 1·85 g. of the hydrocarbon. The suggestion contained in this result of the ability of the rat to metabolise a normal hydrocarbon together with the surprising fact that the hydrocarbon failed to appear in the liver but did apparently find its way to the tissues, led us to investigate the matter further. Our first object was to confirm and extend these results by use of an animal which would provide more material for investigation, and also to study the distribution of the hydrocarbon in the tissues by its isolation in chemically pure form.

Experimental.

Hexadecane emulsified in a cream made by means of a machine for preparing a "synthetic" cream from butter and milk was administered to a cat. The amounts of hydrocarbon included, negligibly small at the start of the experiment, were gradually increased as the animal became accustomed to consuming the cream. The usual daily food provided—meat and fish—was withheld until the bulk of the cream had been consumed. At the end of each day, the vessel containing the cream residues was removed from the cage, and the contents were quantitatively transferred to a flask by means of ether. The combined residues were stored until the end of the experiment. By this means, 43·70 g. of hexadecane were presented to the cat during 29 days. A control animal received the same quantity of cream without added hexadecane.

Investigation of the tissues. At the end of the experiment and 24 hours after the last meal, the animals were killed by chloroform and skinned. The kidneys, heart, spleen, brain, liver, mesentery, omentum and perirenal fat were then removed. The muscle was then dissected from the skeleton. The tissues were heated with 5 % aqueous sodium hydroxide until dissolution occurred. The solutions were extracted quantitatively with ether. The fat obtained by

evaporation of the ethereal extract was saponified with alcoholic potassium hydroxide, and the alcoholic soap solution was poured into water and again extracted with ether. The crude unsaponifiable fractions were again saponified with sodium ethoxide to free them from possible contamination with fat, in view of the large amounts of some of the tissues used as starting material. The yields of unsaponifiable matter, together with its cholesterol content and iodine value, and the calculated iodine value of the non-sterol fraction of the unsaponifiable matter from the animal which received hexadecane are recorded in Table I.

Table I.

	Wt. of tissue g.	Unsap. matter g.	Unsap. matter %	Iodine value	Cholesterol as % of unsap. matter	Iodine value of non-sterol material (calculated)
Kidneys	56	0·2882	0·515	91·5	59·7	129·6
Heart	15	0·0827	0·551	42·4	20·0	36·5
Spleen	14	0·0919	0·656	57·1	58·9	44·6
Brain	26	0·6306	2·425	64·2	86·7	53·9
Liver	155	1·7943	1·158	66·5	58·9	67·4
Mesentery	56	0·4001	0·715	19·3	13·3	12·1
Omentum	176	1·0881	0·618	26·3	8·9	27·6
Perirenal fat	107	1·1313	1·057	28·0	13·3	22·2
Muscle	1531	3·2235	0·215	32·2	27·3	19·6
Skin	934	4·7691	0·511	23·5	26·1	8·6

These figures show that in the majority of these fractions the percentage of cholesterol in the unsaponifiable material is low, and this suggests the presence of excess of some unusual non-sterol constituent. That this is a saturated compound appears from the very low iodine values of the non-sterol fractions, particularly of the unsaponifiable materials of the mesentery, omentum, perirenal fat, muscle and skin which are 12·1, 27·6, 22·2, 19·6 and 8·6 respectively. The unsaponifiable fraction of the omentum was a deep yellow oil, those of the muscle and perirenal fat were almost liquid at room temperature, while the materials from the mesentery and skin were low-melting solids. In view of the small amounts of materials available, isolation of any hexadecane present could only be attempted in the case of the omentum, muscle, perirenal fat, skin and liver. These fractions were submitted to distillation *in vacuo* in Claisen flasks of suitable size. At the end of the distillations, the flasks were cooled and the residues quantitatively transferred by ether to small flasks, dried at 100° *in vacuo* and weighed. Because of its high cholesterol content (58·9 %), the liver material was dissolved in light petroleum and much of the cholesterol removed by crystallisation at 0° before distillation. The remainder of the cholesterol was quantitatively removed by means of digitonin. In Table II, the results of the

Table II. *Distillation of the unsaponifiable fractions.*

	Unsap. matter g.	Distillate g.	Yield as % of unsap. matter	Residue g.	Wt. of distillate referred to the whole of each unsap. fraction g.
Omentum	0·541	0·259	47·9	0·284	0·521
Muscle	2·49	1·54	61·8	0·900	1·99
Perirenal fat	0·681	0·329	48·3	0·360	0·547
Skin	4·21	1·27	30·2	2·91	1·44
Liver*	0·619	Trace	—	0·609	—
					4·5 g

* Sterol-free.

distillations are recorded, and the yields of distillate in the whole of each unsaponifiable fraction are shown in the last column.

The distillates were all obtained over a range of 152–166° at 18–21 mm. The hexadecane administered had B.P. 152°/12 mm., and bearing in mind the difficulties of distilling small amounts of materials such as these, the distillates could be considered as reasonably homogeneous. All the corresponding unsaponifiable fractions from the control cat were submitted to an identical distillation procedure, but in no case was any distillate obtained. Analysis of these fractions gave the following figures: Skin: C, 85·6; H, 14·47 %. Omentum: C, 84·23; H, 15·06 %. Muscle: C, 85·35; H, 14·52 %. Perirenal fat: C, 84·90; H, 14·98 %. $C_{16}H_{34}$ requires: C, 84·85; H, 15·15 %. These analytical figures indicated that all the fractions were fairly pure hexadecane. The fractions were therefore mixed, transferred quantitatively to a test-tube and treated twice with an equal bulk of concentrated sulphuric acid at 130° for 15 minutes with constant shaking to remove any contaminating oxygen compounds. The oil was then frozen out by cooling to 0°, transferred to a funnel with ether and washed, first with dilute alkali to remove sulphuric acid and sulphonic acids, and finally with water until the washings were neutral. After removal of the solvent the residue was dried at 100° *in vacuo*; 2·70 g. colourless oil were obtained having the following constants:

	M.P.	B.P.	Mol wt. (Rast)	C	H	Δ_4^{24}	Δ_4^{18}
Found	18	288	230, 234	84·73	14·97	0·772	—
Required for $C_{16}H_{34}$	18	288	226	84·85	15·15	—	0·775

These data show that the 2·70 g. of purified material was hexadecane. Unavoidable losses occurred in the treatment with sulphuric acid, but the isolation of the greater part of the material in chemically pure form and the carbon and hydrogen figures on the crude distillates leave little doubt that the latter were almost entirely hexadecane.

The amount of hexadecane absorbed.

Preparation of the unsaponifiable fraction from the cream residues yielded 12·06 g. of a pale yellow mobile liquid. Treatment with sulphuric acid by the method already described yielded 11·04 g.

The faeces, which had been collected throughout the experiment and stored under alcohol, were worked up for their unsaponifiable matter by the method used by Mahdi and Channon [1933]. The yield was 7·88 g. of a brown liquid which had an iodine value of 12·2 and contained 26·6 % of sterol precipitable by digitonin. The residue, which weighed after analysis 7·4213 g., was subjected to three successive treatments with sulphuric acid and yielded finally 3·6 g. of hexadecane. This was equivalent to 3·81 g. from the whole of the faecal excretion. Thus of 43·70 g. of hexadecane presented to the animal, 12·06 remained in the unconsumed food and 3·81 g. were excreted. The cat thus absorbed 27·83 g. of the hydrocarbon in 29 days, or approximately 1 g. per day.

Discussion.

These results confirm and extend those previously obtained on rats. The failure to isolate the hydrocarbon from the liver is of interest, and there seems little doubt that it was present, if at all, in traces only. The quantitative removal of the sterol before distillation of the liver-unsaponifiable fraction was carried out in order to facilitate the isolation of any hydrocarbon present, and yet the

0·619 g. of sterol-free material yielded on distillation only a trace of an oil, too small an amount for investigation. Further, 0·541 g. of the material from the omentum, which still contained its sterol, readily yielded 0·259 g. of hexadecane on distillation. This seems therefore proof of the absence of the hydrocarbon from the liver. With this result may be contrasted those previously obtained with squalene [Channon, 1926], and with a medicinal liquid paraffin [Channon and Collinson, 1929]. In the former experiments, squalene was isolated from the livers of rats, and in the latter, definite though indirect evidence of the presence of liquid paraffin in the livers of rats was obtained, and this was confirmed by the isolation of about 1 g. of the paraffin from the liver of a pig which had received the paraffin for a period of 54 days.

Hence the unsaturated hydrocarbon squalene, and a liquid paraffin which was shown to be a mixture of polycyclic hydrocarbons, both appear in the liver when administered in the diet, whereas the normal aliphatic hydrocarbon hexadecane does not. It is difficult to interpret the significance of this finding, especially as work on the fat-soluble vitamins and allied substances of recent years has shown that the liver appears to be the tissue in which these substances are predominantly stored. Equally difficult of interpretation is the presence of the hexadecane in the other tissues. Although sufficient material was not available for its isolation from all the tissues, inspection of Table I makes it appear not impossible that it may have been present in all of them. Be that as it may, hexadecane constituted from 30·2 to 61·8 % of the unsaponifiable fraction of the omentum, muscle, perirenal fat and skin, with an average of 47 %. Hence hexadecane does not appear in the liver, but does appear in the tissues. One point worthy of notice is that if the results are referred to yields of hexadecane per kg. of fresh tissue, they become: perirenal fat 3·08 g., omentum 1·47 g., skin 1·36 g. and muscle 1·00 g. per kg. The result with muscle is rendered uncertain, however, because the pad of fat ventral to the xiphisternum was included with the muscle tissue. Thus, so far as these results can be interpreted at present, they suggest provisionally that the hydrocarbon is distributed in the tissues in proportion to the amount of fat which the tissue contains.

We have no positive evidence as to whether squalene and liquid paraffin, which do appear in the liver on administration, also find their way into the fat depots, and we are giving attention to this problem. If the suggestion that the concentration of hexadecane in the tissues depends on their fat content is correct, it would be in keeping with its absence from the liver, which contains an amount of neutral fat of the order of 1 % only. The unsaponifiable fractions of depot fat are so small in amount that they have been little investigated, and these results suggest that they would repay further study.

Of the 28·14 g. of hexadecane absorbed, 4·50 g. only were isolated from the tissues, and hence 24·74 g. were metabolised in 29 days. As to the intermediate steps involved in this process we have no information at present, and we are investigating this problem further by another line of approach. No positive information is as yet available as to how the body finally metabolises any of the unsaponifiable substances. The general tendency for such substances to appear in the liver may suggest that that tissue plays a predominant part in their metabolism, as it does in the case of the carotene-vitamin A conversion. On the other hand, the latter may be a process peculiar to the liver which may act merely as a temporary place of storage for unsaponifiable substances generally. If the liver were concerned in the breakdown of these substances, it is conceivable that its ability to deal with a chemically inert material such as an aliphatic hydrocarbon is limited. The accumulation in the liver of the amounts

of hexadecane found in the tissues of the cat used in this experiment could obviously not be allowed to occur, if for no reason other than that of the solvent action of such quantities of the hydrocarbon on the lipoid constituents of the liver cells.

SUMMARY.

1. A cat absorbed 28·14 g. of *n*-hexadecane when 33·64 g. were administered to it in cream over a period of 29 days.

2. Consideration of the iodine values and cholesterol contents of the unsaponifiable fractions prepared from the different tissues suggested that hexadecane was generally present in them.

3. The hydrocarbon was isolated in chemically pure form from the omentum, perirenal fat, muscle and skin; it could not be isolated from the liver. Its distribution in the tissues appears to be related to their fat content.

4. The relationship of these findings to those previously obtained in experiments with squalene and with a liquid paraffin which was a mixture of polycyclic hydrocarbons is discussed.

5. The animal can metabolise *n*-hexadecane.

REFERENCES.

Channon (1926). *Biochem. J.* **20**, 400.
—— and Collinson (1929). *Biochem. J.* **23**, 676.
Mahdi and Channon (1933). *Biochem. J.* **27**, 1487.

LXVII. SOME FACTORS WHICH AFFECT THE ASSAY OF VITAMIN A BY THE SPECTROGRAPHIC METHOD.

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(*Received January 13th, 1934.*)

THE physical methods of vitamin A assay have received much attention in the last few years but the trustworthiness of these determinations is still not clearly defined. Before discussing the present work, which deals with one aspect of the estimation of the vitamin, it may be of value to summarise briefly the present position.

The characteristic absorption spectrum of vitamin A is considered to be a broad continuous band with its head at 3280 Å. The intensity of the absorption at this wave-length in oils containing the vitamin has been found to agree very satisfactorily with the biological estimations [Coward *et al.*, 1931; 1932; Chevalier and Chabre, 1933]. This implies that constituents of the oil other than vitamin A absorb a proportional amount of light at 3280 Å.—or are transparent.

The blue colour obtained with the antimony trichloride reagent has been the subject of a large volume of work, and it now appears that the colour measured in the tintometer is the effect of two absorption bands, one at 5720 Å. and one at 6060 Å., whose positions are shifted by approximately 120 Å. towards the red in concentrated specimens. The intensities of these two bands have been compared with the intensity of absorption at 3280 Å. of the oil itself by Coward *et al.* [1931] and Gillam and Morton [1931], and it has been shown that while the intensities of the 5720 Å. band (in the antimony trichloride colour) and the ultra-violet band (in the oil itself) are always similar, the intensity of the 6060 Å. band does not appear to be so related. Furthermore, on oxidative treatment the latter band may be augmented in intensity leaving the other unaffected. As a result of their experiment, Heilbron *et al.* [1931] have suggested that only the band at 5720 Å. in the colour test is valid as a measurement of vitamin A activity.

The conditions which influence the intensities of these two bands of the colour are important in considering the validity of the blue test. The 6060 Å. band may be increased in intensity by keeping, by mild oxidative treatment with ozone, hydrogen peroxide and benzoyl peroxide and by saponification. It may be decreased in intensity by the addition of the antimony trichloride reagent in two stages, by the addition of pyrrole, indole, skatole or an unsaturated acid isolated from the saponifiable fraction of cod-liver oils [v. Eekelen *et al.*, 1933; Heilbron *et al.*, 1931]. The evidence points to the possibility of inhibition of the 6060 Å. band, but the improbability of inhibition of the 5720 Å. band. Assuming that the 5720 Å. band arises from vitamin A, it is necessary to adjust the conditions of conducting the "blue test" in order that the 6060 Å. band shall always bear the same relation to the 5720 Å. band.

For untreated cod-liver oils this appears to be the case, the ratio 6060 Å. band : 5720 Å. being 1·35 : 1, though considerable fluctuation about this mean value occurs (Gillam and Morton). If the absorption at 6060 Å. is caused to attain its maximum value by oxidative treatment a rather more constant ratio is attained (Heilbron *et al.*), but the mean value of the figures given the present writer finds to be 1·45 : 1 which differs from the previous ratio quoted by less than the estimated experimental error. This appears to the writer to indicate that in many cod-liver oils there can be very little inhibitor substance affecting the 6060 Å. band. In these oils saponification should not increase materially the absorption of the 6060 Å. band. In those cases where this band may be increased, we must infer that the ratio is less than about 1·40 : 1 and in order to attain the constant ratio, some means (*e.g.* saponification) must be used to enhance the 6060 Å. band. From these data it appears that in order that the colour test may measure variations in the 5720 Å. band, the determination must be made on the unsaponifiable matter in order that a constant ratio between the two bands of the colour test may be attained.

There is, however, another aspect of the problem. v. Eekelen *et al.* [1932; 1933], have stated that by an adsorption technique it has been possible to separate the two chromogens of cod-liver oils, which give rise to the two bands of the colour test. Only the fraction giving rise to the 6060 Å. band has been found to be active biologically. These experiments are at variance with the more circumstantial evidence which has been already summarised. The conclusions do not in any way affect the technique of making colour tests, however, since in each case the aim must be to cause the 6060 Å. band to attain its maximum value.

Measurements of vitamin A content on the basis of absorption at 3280 Å. involve, however, a purely empirical assumption that irrelevant absorption at this wave-length bears a constant relation to the relevant absorption for all oils or is negligible in comparison with it. With a view to attempting to estimate this irrelevant absorption, cod-liver oils have been aerated at 100° and the absorption determined at intervals. At the same time a sample of the oil was saponified and the absorption of the unsaponifiable material determined. In Fig. 1 a typical curve is shown for an oil of blue value 22 (B.P. test) referred to 20 % solution. Curve I which refers to the absorption at 3280 Å. of the oil on aeration may be regarded as compounded of two curves. By producing back the straight portion of the curve to the axis representing absorption and subtracting the two curves, curve III is obtained. From curve II which represents the absorption of the unsaponifiable matter after the oil has been aerated for varying times (referred to 1 % of oil) it is seen that the absorption attained a constant value after 5 hours, by which time no blue colour was obtained with antimony trichloride. This constant value may be taken as representing the absorption of unsaponifiable matter which is not vitamin A, and by subtracting it from curve II the true values for vitamin A should be obtained. This last curve is almost

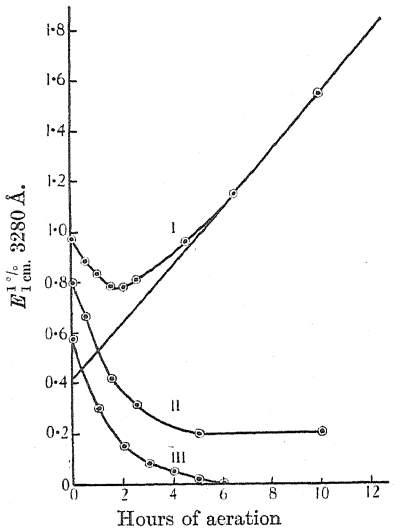

Fig. 1.

identical with curve III which is logarithmic. It is, therefore, assumed that the extrapolated straight line in curve I represents the manner in which the absorption at 3280 Å. of the non-relevant part of the oil varies with time of aeration. If this is true, the intercept of this line on the axis representing $E^{1\%}_{1\,cm.}$ provides a value for the absorption of the constituents other than vitamin A in the original oil, and in this case it amounts to approximately 40 % of the total absorption. The curves shown draw attention to the possibility of serious inaccuracies arising from the measurement of absorption at 3280 Å. as the sole criterion of vitamin A potency. Furthermore, the effect of previous ageing of the oil by oxidation probably has the effect of increasing the irrelevant, as well as decreasing the relevant, absorption at 3280 Å. The actual variation in absorption by oxidative change at room temperatures cannot of course be gauged by these curves since the temperature coefficients of the two reactions involved will probably be different. From the published figures of Heilbron *et al.* [1931] it appears that in some cases at least an actual increase of total absorption may occur after storing.

In Fig. 2 two further curves of aeration are given, curve I for an unusually potent cod-liver oil of blue value 112, and curve II for a weak oil of blue value 14. In each case a similar curve to that in Fig. 1 has been obtained, the linear

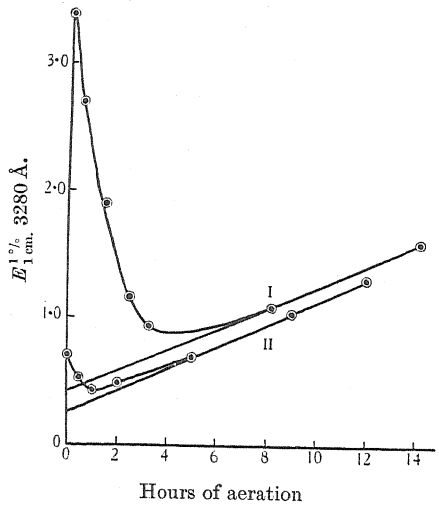

Fig. 2.

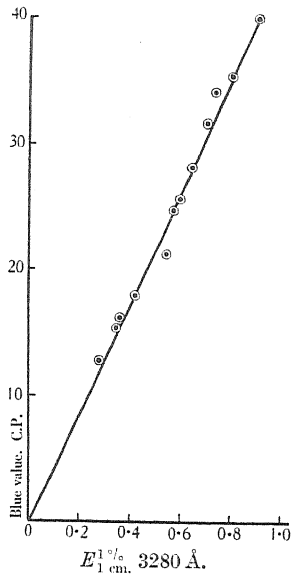

Fig. 3.

portions of which are approximately parallel and of similar initial values. Again, in the less potent oil the irrelevant absorption is of the order of 40 %, while in the potent oil it is approximately 12 %.

In a recent paper Dann and Moore [1933] have shown that the time of saponification is a factor which affects the absorption of the fatty acids from cod-liver oils. Edisbury *et al.* [1933] have suggested "cyclisation" as the cause. While a discussion of the chemical nature of the changes brought about by aeration treatment lies outside the scope of this paper, it is suggested that the increase in absorption at 3280 Å. by aeration may arise from a similar cause since the absorption at lower wave-lengths is increased very considerably, but by the

technique used it would not have been possible to detect the fine structure bands noted by these authors.

It would seem that the absorption at 3280 Å. can only be regarded as measuring the vitamin A activity for oils more potent than that shown in Fig. 2, curve I. For less potent oils the "non-vitamin A" absorption at 3280 Å. constitutes a considerable proportion of the total absorption. The very real parallelism, however, between the absorption in this region and the biological tests for normal cod-liver oils cannot be dismissed as fortuitous. It has been found that the oils which have been so far examined by the aeration technique contain approximately 40 % of irrelevant matter, as measured by the intercept, provided that the potency is of the order of 10–20 blue units. In these circumstances the measured absorption of the oil will be proportional to the amount of vitamin A present.

In Fig. 3, a curve for the blue values and absorption values of the unsaponifiable fraction of cod-liver oils has been drawn. Of all the oils which have been examined none has deviated from this curve by more than 12 %. Josephy [1933] has recorded some results of a similar nature.

While it is not practicable to adopt the aeration technique here described, it appears to the author desirable to determine the absorption upon the unsaponifiable matter from the oil rather than upon the oil itself. In this way errors arising from previous oxidation of the oil are eliminated and there only remains the error due to absorption at 3280 Å. of irrelevant unsaponifiable matter.

Summary.

The effect of oxidative change on the absorption at 3280 Å. of liver oils is discussed in so far as it affects the assay of vitamin A by the spectrographic method. The need for saponification of the oil in order to avoid errors arising from previous oxidation is emphasised.

The author is indebted to Prof. Drummond for his interest in the work and his helpful suggestions.

REFERENCES.

Chevalier and Chabre (1933). *Biochem. J.* **27**, 298.
Coward, Dyer and Morton (1932). *Biochem. J.* **26**, 1593.
—— —— —— and Gaddum (1931). *Biochem. J.* **25**, 402.
Dann and Moore (1933). *Biochem. J.* **27**, 1166.
Edisbury, Morton and Lovern (1933). *Biochem. J.* **27**, 1451.
v. Eekelen, Emmerie, Julius and Wolff (1932). *Kon. Akad. Weten. Amsterdam*, **35**, 1347.
—— —— —— —— (1933). *Nature*, **132**, 171.
Gillam and Morton (1931). *Biochem. J.* **25**, 1346.
Heilbron, Gillam and Morton (1931). *Biochem. J.* **25**, 1352.
Josephy (1933). *Acta Brev. Neerl.* **3**, 133.

LXVIII. THE ENZYMIC HYDROLYSIS OF PHOSPHATIDES.

III. THE HYDROLYSIS OF NATURAL AND SYNTHETIC PHOSPHATIDES.

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It has previously been shown [King, 1931] that an enzyme is contained in the kidney and intestinal mucosa which is capable of hydrolysing lecithin to liberate inorganic phosphate at an optimum p_H of 7·5. The enzyme appears to be present in other tissues to only a slight extent. Bone, which is very rich in an enzyme hydrolysing primary esters of phosphoric acid, is relatively feeble in its action against lecithin. Lecithinase appears to act most rapidly at body temperature (Fig. 1). It is fairly stable at a neutral reaction but is rapidly destroyed in an alkaline or acid medium. The concentration of substrate (Fig. 2) appears to

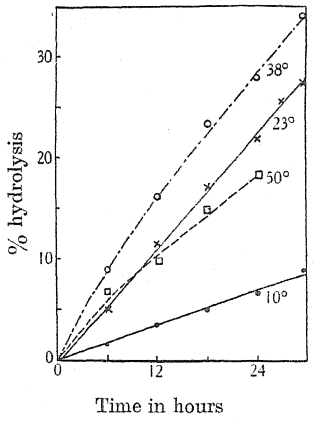

Fig. 1. Effect of temperature on the hydrolysis of lecithin:

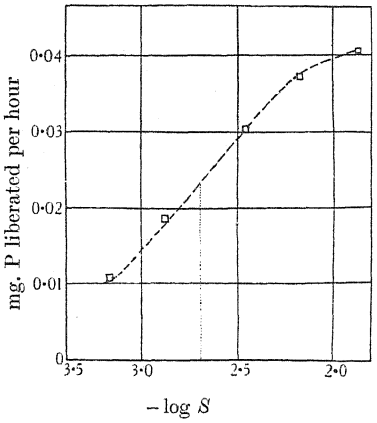

Fig. 2. Effect of substrate concentration on lecithinase hydrolysis:

10 ml. of lecithin emulsion at p_H 7·5 + 2 ml. of dialysed extract of intestinal mucosa.

affect the hydrolysis in a manner similar to that indicated by Michaelis and Menten [1913]. Owing to the slowness with which lecithin is broken down by the enzyme, it has been impossible to obtain any accurate measure of the initial velocity of hydrolysis. The results appear to indicate, however, that the substrate concentration giving half the maximum velocity corresponds to about 0·002 M ($-\log S$ 2·7), which is nearly the same as that found by Martland and

Robison [1927] for phosphatase, *i.e.* $<0{\cdot}003\,M$ [cited by Haldane, 1930]. It is the purpose of the present paper to record the results of a study of the action of this enzyme on other phosphatides, both natural and synthetic.

Lecithin, kephalin and phosphatidic acid.

Liver-lecithin behaved much like egg-lecithin, while kephalin showed a lower rate of hydrolysis with not so marked a dependence on hydrogen ion concentration. A sample of phosphatidic acid from cabbage leaf [Chibnall and Channon, 1927] was kindly supplied to us by Prof. A. C. Chibnall. The formula of phosphatidic acid is represented as corresponding with that of lecithin with the choline absent. It showed a fairly high rate of hydrolysis, but the relation of the amount of hydrolysis to the p_H was not definite as in the case of lecithin (see Table I).

Table I. *Hydrolysis of natural phosphatides by extract of the small intestine.*

(mg. inorganic phosphorus.)

5 ml. borax-boric acid or borax-succinic acid buffer, 5 ml. 0·25 % emulsion of phosphatide and 2 ml. enzyme solution. The hydrolysis was carried out in the presence of chloroform and toluene for 12 hours at 37·5° and was stopped by the addition of 2 ml. 25 % trichloroacetic acid. The inorganic phosphate in the filtrates was estimated by the colorimetric method.

	p_H	Control (1); buffer + phosphatide	Control (2); buffer + enzyme	Test; buffer + phosphatide + enzyme	Phosphate hydrolysed
Egg-lecithin	5·5	0·000	0·041	0·058	0·017
	6·5	0·000	0·037	0·082	0·045
	7·1	0·000	0·038	0·109	0·071
	7·5	0·000	0·039	0·119	0·080
	8·1	0·000	0·039	0·112	0·073
	8·9	0·000	0·038	0·091	0·053
	9·2	0·000	0·037	0·068	0·031
Liver-lecithin	5·6	0·000	0·040	0·056	0·016
	6·5	0·000	0·037	0·067	0·030
	7·1	0·000	0·038	0·091	0·053
	7·6	0·000	0·039	0·096	0·057
	8·0	0·000	0·039	0·095	0·056
	8·9	0·000	0·038	0·066	0·028
	9·2	0·000	0·037	0·056	0·019
Kephalin	4·3	0·020	0·035	0·069	0·014
	4·8	0·020	0·034	0·070	0·016
	5·0	0·021	0·034	0·083	0·028
	5·5	0·020	0·041	0·103	0·042
	6·5	0·021	0·037	0·103	0·045
	7·1	0·020	0·038	0·101	0·043
	7·6	0·020	0·039	0·101	0·042
	8·3	0·019	0·039	0·107	0·049
	8·9	0·020	0·038	0·090	0·032
	9·2	0·021	0·036	0·089	0·032
Phosphatidic acid	5·6	0·068	0·040	0·191	0·083
	6·6	0·069	0·037	0·193	0·087
	7·0	0·067	0·038	0·187	0·082
	7·5	0·067	0·039	0·185	0·079
	8·0	0·070	0·038	0·185	0·077
	8·9	0·073	0·038	0·185	0·074
	9·2	0·069	0·037	0·180	0·074

Lysolecithin.

Lysolecithin was prepared by the action of the venom of the fer de lance on the lecithin contained in egg-yolk [King and Dolan, 1933]. The material so prepared has marked haemolytic properties. The behaviour of lysolecithin with

both the intestinal and bone enzymes was investigated. The amounts of hydrolysis at different hydrogen ion concentrations and at different times, when lysolecithin was subjected to the action of an aqueous extract of rabbit intestinal mucosa, indicated that the maximum hydrolysis occurred at about p_H 7·8, which is a little higher than the corresponding value for lecithin, and that this relatively simpler phosphatide was hydrolysed about twice as fast as the parent lecithin.

Bromolecithin.

Bromolecithin was prepared by passing bromine vapour in a stream of carbon dioxide into a mixed ether and light petroleum solution of egg-lecithin and liver-lecithin [Levene and Rolf, 1926]. The precipitated material was purified by solution in ether and reprecipitation by light petroleum.

Analysis for phosphorus was made by the perchloric acid colorimetric procedure [King, 1932], for nitrogen by micro-Kjeldahl, and for bromine by the potash fusion method of Fendler and Stüber [1914], the silver bromide being collected by means of a porcelain filter stick. (Found: P, 2·02; N, 1·04; Br, 41·3 %. Calculated: for octabromoarachidyl-stearyl-lecithin: P, 2·12; N, 0·96; Br, 43·7 %; for octabromoarachidyl-palmityl-lecithin: P, 2·16; N, 0·97; Br, 44·6 %; for hexabromostearyl-stearyl-lecithin: P, 2·42; N, 1·09; Br, 37·5 %.)

The behaviour of bromolecithin in the presence of phosphate-liberating enzymes was in marked contrast to that of the lecithin from which it had been made. With a variety of enzyme preparations from several sources, bromolecithin was practically completely hydrolysed in 24 hours, while lecithin was attacked only to the extent of 20 % in the most favourable instance. This peculiar property of greatly increased susceptibility to enzymic hydrolysis resulting from bromination of lecithin is probably related to an increased affinity of the enzyme for the phosphatide, due to the change in the fatty acid side-chain. The effect of p_H on the enzymic hydrolysis of bromolecithin is shown in Fig. 3.

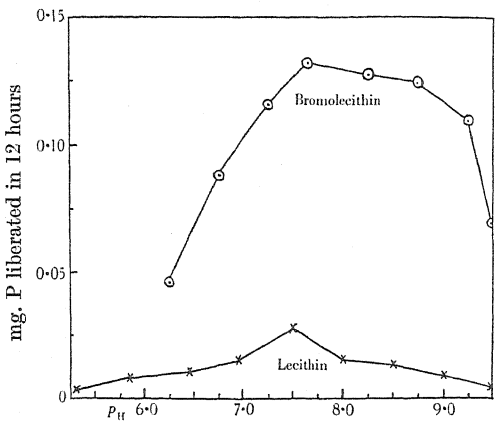

Fig. 3. Effect of p_H on hydrolysis of bromolecithin by cat-kidney extract. Each test contained 0·16 mg. of phosphatide-P, and 0·5 ml. of enzyme. Veronal-acetate buffer; hydrolysis at 38° for 12 hours.

The rates of hydrolysis of bromolecithin, lysolecithin and the parent lecithin are given in Table II, the hydrolysis constant being calculated from $k=\frac{1}{t}\log\frac{a}{a-x}$. The velocity of hydrolysis for bromolecithin was about 10 times as great as for

lecithin in the case of the intestinal enzyme, and 100 times as great with bone-phosphatase. The bone-enzyme, moreover, gave with bromolecithin about twice the velocity of hydrolysis that was obtained with the intestinal enzyme. This is in marked contrast to the action of the two enzyme preparations on lecithin. The velocities for bromolecithin were in about the same ratio as the phosphatase activities of the two preparations measured against glycerophosphate.

Table II. *Comparative rates of enzymic hydrolysis of phosphatides.*

	Lecithin		Lysolecithin		Bromolecithin	
Hours	% hydrolysis	k	% hydrolysis	k	% hydrolysis	k
	(1) Rabbit intestinal extract.					
½	1·0	0·0072	1·6	0·0138	9·1	0·0834
1	1·2	0·0054	2·0	0·0090	17·9	0·0860
2	2·4	0·0054	2·4	0·0054	29·0	0·0744
4	3·2	0·0036	4·8	0·0054	44·8	0·0645
7	4·8	0·0031	7·9	0·0052	59·9	0·0567
	Average	**0·0049**		**0·0078**		**0·0750**
	(2) Rat bone extract (phosphatase).					
½	—	—	0·7	0·0070	15·1	0·1424
1	0·4	0·0018	0·8	0·0036	28·6	0·1461
2	0·8	0·0018	1·1	0·0025	50·0	0·1505
5	1·2	0·0010	1·3	0·0012	65·9	0·0934
7	1·5	0·0009	1·6	0·0010	70·2	0·0751
	Average	**0·0014**		**0·0031**		**0·1215**

Synthetic phosphatides.

Through the kindness of Dr Adolf Grün, of the Hoffman-La Roche Company, Grenzach, Germany, we were able to compare samples of the synthetic phosphatides described by Grün and Limpächer [1927]. Synthetic distearyllecithin did not show the same general effect on enzymic hydrolysis as did the natural lecithin (Table III). The rate of hydrolysis was not so great and the p_H effect

Table III. *Influence of* p_H *on the enzymic hydrolysis of natural and synthetic phosphatides.*

(1) Natural egg-lecithin		(2) Synthetic lecithin (Grün)		(3) Distearyl phosphate (Grün)	
p_H	mg. P liberated	p_H	mg. P liberated	p_H	mg. P liberated
3·6	0·145	5·7	0·121	4·5	0·183
4·3	0·158	6·2	0·115	5·9	0·193
5·9	0·186	6·9	0·119	6·6	0·176
6·9	0·221	7·3	0·121	6·9	0·178
7·1	0·220	7·5	0·118	7·4	0·174
7·3	0·222	8·0	0·111	7·8	0·176
7·5	0·205	8·8	0·127	8·2	0·170
7·8	0·192			9·5	0·145
8·1	0·170				

was absent. It was thought that this difference might be due to the unsaturation and greater solubility of the natural product. This explanation was found to be untenable, however, by the discovery that hydrogenated lecithin has almost the same rate of enzymic hydrolysis as has the lecithin from which it is made (Table IV).

Synthetic distearyl phosphate showed very little relation between the p_H and the amount of hydrolysis. Since it is a primary ester of phosphoric acid,

Table IV. *Enzymic hydrolysis of hydrolecithin and lecithin.* (*mg. P liberated.*)

Tests consisted of 2 ml. of enzyme (rat-intestinal extract), 5 ml. of veronal buffer and 5 ml. of emulsified phosphatide containing 0·086 mg. of hydrolecithin-P per ml. and 0·088 mg. of lecithin-P per ml. The hydrolysis took place at 38° for 48 hours.

p_H	Hydrolecithin*	Lecithin	p_H	Hydrolecithin*	Lecithin
5·8	0·114	0·114	8·0	0·203	0·194
6·5	0·151	0·158	8·5	0·187	0·188
7·1	0·208	0·206	8·9	0·161	0·170
7·5	0·201	0·202	9·4	0·052	0·082

* This sample of hydrolecithin was kindly given to us by Dr P. A. Levene. Other samples prepared in this laboratory by hydrogenation of egg- and liver-lecithins in the presence of palladium gave very similar results.

it might have been expected to behave like glycerophosphoric acid or acetonylglycerophosphoric acid, which are hydrolysed at an optimum p_H of 8·9, whereas lecithin, a secondary ester of phosphoric acid, is hydrolysed at an optimum p_H of 7·4 (diphenyl phosphate and diethyl phosphate are hydrolysed at a slightly lower p_H optimum). Comparative figures for the hydrolysis of natural and synthetic lecithin and distearylglycerophosphoric acid were obtained by incubation of 5 ml. of 0·25 % emulsion of the phosphatide mixed with an equal volume of buffer at several p_H values and 2 ml. of the enzyme solution for 48 hours. The amount of phosphate liberated from the egg-lecithin was considerably greater than that from the distearyl phosphoric acid and at the optimum p_H was almost double that liberated from the synthetic lecithin.

Discussion.

The extraordinary change induced in lecithin by bromination is not easily explained. It is a more common experience to find that a large increase in molecular weight (that of bromolecithin is almost double that of lecithin) leads to a decrease rather than an increase in reactivity. A possible explanation of this anomalous result of greatly increased susceptibility to enzymic hydrolysis may lie in a greater negativity of the alcohol part of the ester due to introduction of several bromine atoms into the side-chain. Among the simple primary esters of phosphoric acid those containing the more negative alcohol groups are most readily attacked by phosphatase; thus phenyl phosphate is much more rapidly hydrolysed than tolyl phosphate, and tolyl phosphate very much more rapidly than ethyl phosphate.

In the case of phosphatidic acid, the p_H effect was almost absent, and since phosphatidic acid is a primary phosphoric ester, it—as also distearyl phosphate—might have been expected to be hydrolysed optimally at p_H 8·9. Since, however, the phosphate-liberating enzyme is relatively unstable above p_H 8, while it is quite stable in more neutral solutions, the discrepancy may be explicable on the basis of destruction of the enzyme during the course of hydrolysis. If this be so, the length of the fatty acid chains may be the determining factor, since it probably conditions the solubility of the phosphatide and its consequent availability for enzymic hydrolysis. The shorter the carbon chains of the substituent groups, the more soluble the substance should be and the more it should behave like a simple primary phosphoric ester with a p_H optimum of 8·9. It is also possible that the nature of the fatty acid radicals may affect, through some mechanism akin to steric hindrance, the union of substrate and enzyme.

Thus the presence of the fatty acids in phosphatidic acid may hinder its union with the enzyme, giving a lower rate than would be obtained with an unsubstituted glycerophosphate, or with a glycerophosphate substituted with less complex groups than stearic and oleic. This, combined with destruction of the enzyme, might account for the failure of this product to be hydrolysed at the optimum p_H of 8·9.

If the difference between the behaviours of natural and synthetic phosphatides on enzymic hydrolysis were due to the unsaturation of the natural product, then phosphatidic acid, which is more unsaturated than egg-lecithin, might have been expected to show a higher rate of hydrolysis. Also, hydrolecithin, which ought to be very similar in chemical behaviour to the synthetic product if existing theory as to the structure of these substances be correct, might have been expected to behave like synthetic lecithin, instead of which it showed an enzymic hydrolysis almost identical with that of its parent lecithin. These facts would appear to cast some doubt on the similarity of the chemical configuration of the synthetic lecithin of Grün and Limpächer with the naturally occurring phosphatide; and further, if the chemical formula assigned by Grün and Limpächer to the synthetic product be correct, then the formula commonly used to represent the chemical structure of lecithin may be in error. The asymmetric formula which appears in most of the textbooks and represents lecithin as always being a derivative of α-glycerophosphoric acid, has been shown to be incorrect [Rae, 1934], and it is possible that a more drastic revision of the manner of representing the formula for lecithin may yet be necessary.

SUMMARY.

An enzyme is contained in kidney and intestinal mucosa which hydrolyses lecithin from several sources with the liberation of phosphoric acid. Hydrolecithin is attacked as fast as its parent lecithin, kephalin and phosphatidic acid somewhat more slowly, the latter without showing any marked dependence on p_H. Synthetic lecithin and distearyl phosphate are attacked by the enzyme at a lower rate and without showing any p_H optima. Lysolecithin is hydrolysed about twice as fast as lecithin. Brominated lecithin is hydrolysed at a very much greater rate than is lecithin.

The thanks of the author are due to Miss M. E. Dolan and Miss M. M. Shaw for valuable assistance during the course of these experiments.

REFERENCES.

Chibnall and Channon (1927). *Biochem. J.* **21**, 1112.
Fendler and Stüber (1914). *Z. physiol. Chem.* **89**, 123.
Grün and Limpächer (1927). *Ber. deutsch. chem. Ges.* **60**, 147, 151.
Haldane (1930). Enzymes. (Longmans, Green and Co., London.)
King (1931). *Biochem. J.* **25**, 799.
—— (1932). *Biochem. J.* **26**, 292.
—— and Dolan (1933). *Biochem. J.* **27**, 403.
Levene and Rolf (1926). *J. Biol. Chem.* **67**, 659.
Martland and Robison (1927). *Biochem. J.* **21**, 665.
Michaelis and Menten (1913). *Biochem. Z.* **49**, 333.
Rae (1934). *Biochem. J.* **28**, 152.

LXIX. THE ULTRA-VIOLET SPECTRUM OF OXYHAEMOGLOBIN.

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A prominent band in the near ultra-violet spectrum of oxyhaemoglobin was discovered by Soret [1883] who referred to it as the "great" ultra-violet band. Gamgee [1895] pointed out that this band was also found in haemoglobin, carbon monoxide haemoglobin, nitrous oxide haemoglobin, haemochromogen, haematin hydrochloride, methaemoglobin and haematoporphyrin, but was absent from the spectra of bilirubin, hydrobilirubin and urobilin. The position of the peak of this oxyhaemoglobin band in the wave-length scale was found by Rost *et al.* [1909] to be 4100 Å. Newcomer [1919] placed it at 4140 Å. and Schumm [1921] at 4135–4140 Å.

In the case of carbon monoxide haemoglobin the absorption is greater, and the band is shifted towards the red, while the spectrum of acid haematin shows a similar band with the absorption not so pronounced and extending into the ultra-violet, obliterating a small peak at 3650 Å. which is characteristic of the oxy-haemoglobin spectrum.

Oxyhaemoglobin also has two bands with peaks at 3400 Å. and 2700 Å., and beyond 2400 Å. the absorption becomes general. Warburg and Negelein [1929] on measuring the absorption of light by the extent of chemical change which the absorbed energy produced, fixed the peak of the "great" band at 4300 Å. and found another prominent band at 2700 Å. in the case of oxyhaemoglobin, the latter band being lacking in all pure haem compounds.

Newcomer [1919] concluded that the similarity between the spectra indicated the presence of a common radical, but Hicks and Holden [1929] could not find any relationship between the valency of the iron in the pigments and their ultra-violet spectra. Anson and Mirsky [1930] state that pigments of similar chemical structure produce spectra of the same general form and of the same dimensions.

Macallum and Bradley [1930] pointed out that the absorption band occurring at 4100 Å. for crystalline oxyhaemoglobin of the horse does not appear in the spectrum of washed corpuscle suspensions containing the pigment in its normally occurring form. These observations have been verified and extended to the blood pigments of other species.

The instrument employed was a Bellingham and Stanley quartz spectrograph. The ultra-violet spectrum from 2100 to 4500 Å. was extended over 18 cm. upon the negative. The absorption measurements were determined by the use of a photoelectric recording photometer developed in this laboratory by Macallum and Coates [1933].

The blood was drawn from the animal into sodium citrate. In the case of the corpuscle suspensions the blood was diluted with mammalian saline so that the pigment concentration was adjusted to a predetermined value (0·2 % haemoglobin), these values being determined by Newcomer's method. In the case of the haemoglobin, the corpuscle suspension was laked with ether, the haemoglobin values being adjusted as stated above.

Typical results may be illustrated by the data obtained from rabbits' blood (Fig. 1).

1. Unlaked blood. The absorption curve shows a slight elevation about 4500 Å. then a fall towards 4300 Å.; between 4300 and 4000 Å. there is another slight rise terminating about 4000 Å. There is another slight rise at 3900 Å. The curve then becomes level to 3500 Å.

2. Laked blood. The absorption curve is fairly level to 4200 Å. where it rises sharply to a peak about 4100 Å. and then drops off gradually to the original level at 3900 Å. The curve is then level to 3700 Å. where it recedes slightly and then rises at 3600 Å. and comes back to the original level at 3500 Å. The laked blood at the height of the great ultra-violet band (4100 Å.) shows about 40 % absorption for the solution used.

In comparing the laked and unlaked oxyhaemoglobin, it is seen that the laked shows greater absorption at any given wave-length.

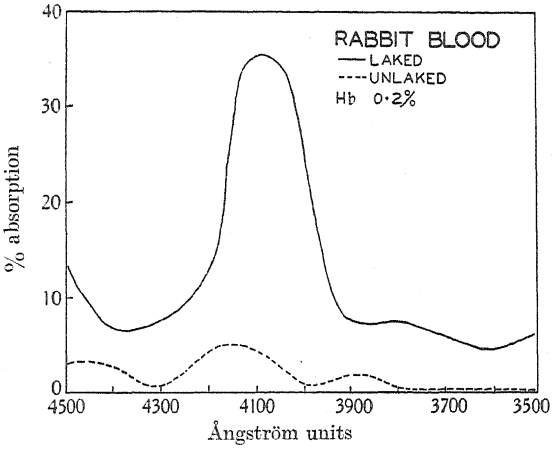

Fig. 1.

Oxyhaemoglobin from other species. For the sake of convenience the beginning of the great ultra-violet band has been designated A, the peak B and the end C, the figures representing Å.

1. Laked blood.

	A	B	C
Rabbit	4200	4100	3900
Goose	4300	4100	3910
Human	4300	4100	3850
Turtle	4300	4100	3900
Horse	4200	4100	3800

2. Unlaked blood. The absorption spectra do not show any marked band. The spectrum of rabbit-oxyhaemoglobin, in the visible region, was examined

both in the corpuscle suspension and in the laked blood and the position of the α and β bands determined. The position of the bands was fixed as follows:

	α band (Å.)	β band (Å.)
Laked blood	5780	5400
Unlaked blood	5780	5400

This experiment was repeated several times on rabbits' blood and indicates that there is no difference in the spectral bands of oxyhaemoglobin inside or outside the corpuscle.

Discussion.

Our observations have been made on oxyhaemoglobin solutions, and they have served to confirm the observations previously recorded. The great ultra-violet absorption curve in the oxyhaemoglobin solutions shows in most cases an origin about 4300 Å., a rise to a peak about 4100 Å. and then a drop back to 3900 Å. The unlaked blood does not show this great ultra-violet band in the region of 4100 Å. The rest of the curve remains much the same except that there is a slight rise in some cases at 4100 Å.; in all cases where this latter absorption occurred there was slight haemolysis, and it is believed that this absorption is due to traces of oxyhaemoglobin outside the corpuscle.

There is the possibility that the absence of the great ultra-violet band from the corpuscle suspensions might be due to the masking effects of the blood-serum, the corpuscle wall, material mixed with haemoglobin in the cell or material combined with haemoglobin in the cell but removed by ether-laking. As to the serum it has been shown by Lewis [1917] that the ultra-violet absorption spectrum of serum has two peaks at 2800 Å. and 2400 Å.; it represents several superimposed curves and does not resemble that of a pure compound, being due chiefly to protein. Furthermore, we have found that the great ultra-violet band of oxy-haemoglobin exists in laked whole blood as well as in aqueous solutions of crystalline oxyhaemoglobin.

The question as to whether the corpuscle wall itself may be a factor can be eliminated by the fact that blood laked by freezing and containing the corpuscle "ghosts" still displays the band in the ultra-violet. Removal of the "ghosts" by centrifuging does not affect the absorption band.

Cell stromata evidently play no part since the laked blood containing corpuscle contents displays the spectrum of pure oxyhaemoglobin.

Finally, since our early experiments were carried out on ether-laked samples it might be possible that this treatment had removed a lipoid fraction which was combined with the oxyhaemoglobin in the corpuscle and that lack of this portion might be responsible for the absence of the band under question. However, the fact that laking brought about by the physical processes of freezing and thawing, which do not remove any constituent of the cell, still gives the same spectrum as the ether-laked blood indicates that the presence of the band is independent of the absence of any ether-soluble substance.

Our results indicate that there is an essential difference between oxyhaemo-globin inside and outside the corpuscle. If the presence of absorption bands is to be interpreted as indicating a condition of desaturation then it must be assumed that oxyhaemoglobin outside the corpuscle is in a state of unsaturation while in its naturally occurring state in the cell it enters into combination with some of the cell constituents. The combination must be rather labile since it is disrupted by any force which alters the osmotic balance of the cell.

REFERENCES.

Anson and Mirsky (1930). *Physiol. Rev.* **10**, 533.
Gamgee (1895). *Proc. Roy. Soc. Lond.* **59**, 276.
Hicks and Holden (1929). *Austr. J. Exp. Biol. Med. Sci.* **6**, 175.
Lewis (1917). *Proc. Roy. Soc. Lond.* B **89**, 337.
Macallum and Bradley (1930). *Science*, **71**, 341.
—— and Coates (1933). *Can. Chem. Met.* **17**, 165.
Newcomer (1919). *J. Biol. Chem.* **37**, 465.
Rost, Franz and Heize (1909). *Arbeiten Kaiserlich. Gesamtheitsamt*, **32**, 223.
Schumm (1921). *Abderhaldens Handbuch Biol. Arbeitsmethoden.* Abt. 4, Teil 3, 116.
Soret (1883). *Compt. Rend. Acad. Sci.* **97**, 1269.
Warburg and Negelein (1929). *Biochem. Z.* **214**, 64.

LXX. THE OXIDATION OF PALMITIC ACID, BY MEANS OF HYDROGEN DIOXIDE IN THE PRESENCE OF A CUPRIC SALT.

PRELIMINARY COMMUNICATION.

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Palmitic is probably the most commonly occurring saturated fatty acid and is one of the fatty acid constituents of lipins; yet, although in the body it must undergo oxidation with extraordinary ease, it is very resistant to the action of the usual laboratory chemical oxidising agents. Even after being heated on a water-bath for 18 hours with a concentrated alkaline solution of potassium permanganate about 40 % of the original palmitic acid was recovered. A dihydroxypalmitic acid and oxalic, succinic and adipic acids were isolated from the reaction product together with acetic, butyric and possibly capronic and caprylic acids [Gröger, 1887]. Information does not appear to be available with regard to palmitic acid but oxidation of myristic acid with strong nitric acid resulted in the formation of a mixture of dibasic acids, amongst which oxalic, succinic, adipic and suberic were identified [Nördlinger, 1886].

A small proportion of palmitic acid was shown by Dakin [1908, 1] to undergo β-ketonic oxidation when its ammonium salt was distilled with hydrogen dioxide on a boiling water-bath. About 10 % of the original acid was thus converted into carbonic acid and methyltridecylketone; the addition of a considerable excess of the dioxide produced very little further effect. Clutterbuck and Raper [1925] showed that the γ- and δ-keto-acids were formed to about the same extent as the β-acid. On further oxidation of the γ- and δ-keto-acids, succinic acid was identified in the product [Clutterbuck, 1927].

Whilst very definite evidence can be obtained that the path of oxidation of the α-amino-acids *in vivo* lies through the α-keto-acids there is no evidence that β-keto-acids are formed as the intermediate products of oxidation of the higher fatty acids. Acetoacetic and β-hydroxybutyric acids accumulate when the mechanism for the oxidation of the fatty acids is overburdened or impaired, and the available evidence points to the β-hydroxy-acid being formed by reduction of acetoacetic acid in the tissues; but these two acids are mutually convertible in the body, and the possibility of hydroxylation being the process involved cannot be excluded.

There is no evidence of the existence in the body of any ketones such as those isolated by Dakin in his experiments on the oxidation of the fatty acids *in vitro*, and if the higher β-keto-acids are so readily oxidised it is difficult to understand why a mechanism which splits the original β-keto-acid so completely into acetic acid and an acid containing two less carbon atoms than the original should cease completely to function when the stage of acetoacetic acid is reached.

The process used in the body is so perfect that no intermediate substances can be identified.

Smedley-MacLean and Pearce [1931] showed that oleic and *n*-octanoic acids were rapidly and almost completely oxidised by the action of hydrogen dioxide in the presence of a cupric salt. In the present investigation the addition of a few drops of cupric sulphate solution to one of sodium palmitate has been found to produce an equally potent effect on the oxidation of palmitic acid by hydrogen dioxide. Contrary to our expectation, palmitic was as readily oxidised as oleic acid. Indeed the behaviours of oleic and palmitic acids when heated for 3 to 4 hours at 90° with a large excess of hydrogen dioxide and a few drops of the cupric solution were very similar. About 70 % of each acid was converted into carbonic and volatile fatty acids. In the earlier stages of the oxidation of palmitic acid volatile lactones giving propionic acid on treatment with strong sulphuric acid were present. Under milder conditions of oxidation, succinic acid was isolated from both oleic and palmitic acids in about the same amounts, and in the case of oleic acid an intermediate monobasic acid, $C_8H_{14}O_4$, was obtained; from its formula this was regarded as a ketohydroxy-acid, though it was not found possible to isolate from it any well-characterised crystalline derivative. The possibility of it being an unsaturated dihydroxy-acid was not excluded and in view of our present results seems probable.

The absence of oxalic or dibasic acids other than succinic acid as intermediate products differentiates the action of hydrogen dioxide from that of other oxidising agents upon palmitic acid. The introduction of the cupric catalyst converts a process of ketonisation into one which appears to consist essentially of hydroxylation and desaturation. At the same time the breaking down of the original acid is enormously facilitated.

Traube [1905; 1906; 1910; 1911; 1932] showed that the catalytic effect of cupric salts on the oxidation of the sugars and phenols was connected with the presence of the hydroxyl group in the molecule, an observation borne out by the extraordinarily rapid oxidation of tartaric acid produced by the dioxide in the presence of a cupric salt [Battie and Smedley-MacLean, 1929].

In the present investigation, the products obtained by continuous extraction with ether of the oxidation product of palmitic acid appeared to be mixtures of isomeric substances in which unsaturated dihydroxy-acids and hydroxylactones predominate. If ketohydroxy-acids are present, they occur only in small quantity and are not the main products of the reaction.

The occurrence of unsaturated acids as oxidation products of the normal higher fatty acids does not appear to have been previously observed. Ricinoleic acid, an unsaturated monohydroxy-acid, is a constituent of castor oil and its presence shows that such an acid may be a normal product of fat metabolism, but whether the ethylene linkage arises by hydroxylation and subsequent loss of water or by dehydrogenation is not known.

From the composition of the products of successive degrees of oxidation, the hydroxyl groups in these acids are probably not adjacent but occur at intervals: their position has however not yet been determined with certainty. From a comparison of the relative numbers of the C and H atoms, as determined by analysis, with the iodine values recorded, which were always lower than the expected values, it is probable that a considerable proportion of the $\alpha\beta$-unsaturated acid was present. Crotonic and cinnamic acids, for instance, give very low iodine values [Smedley-MacLean and Thomas, 1921]. It is possible that β-hydroxylation had taken place with subsequent elimination of water giving a mixture of $\alpha\beta$- and $\beta\gamma$-unsaturated acids.

The formation of a considerable proportion of unsaturated acids as intermediate products during the oxidation of the fatty acids may indicate on the other hand a process of oxygenation which is in part at any rate one of dehydrogenation, and which may be compared with the oxidation of succinic acid in the body brought about by the succinic dehydrogenase: the succinic acid is dehydrogenated forming fumaric acid and the latter adds on water giving malic acid. It is possible that the introduction of the hydroxyl group into the palmitic chain in the presence of copper activates the hydrogen atoms attached to the neighbouring carbon atoms so that they may be removed by the action of the hydrogen dioxide. Dakin has shown that in the body cinnamic acid can arise from phenylpropionic acid and can be converted into γ-phenyl-β-hydroxypropionic acid [Dakin, 1908, 2; 1909], and that the unsaturated and saturated acids gave rise to the same product. The formation of the highly unsaturated acids containing 20 and 22 carbon atoms is also known to be effected by the liver: the identification of unsaturated acids among the oxidation products of palmitic acid in the laboratory appears to offer therefore a better analogy with the method of oxidation used in the body than does the intermediate occurrence of keto-acids.

The isolation of lactones in considerable amount showed that oxidation had taken place in the γ- or δ-position.

Succinic acid was identified at every stage of the oxidation except the earliest. This acid would itself be oxidised to a considerable extent under the conditions of the experiment, but even allowing for this the total proportion of succinic acid formed would correspond to considerably less than one molecule of succinic acid for each molecule of palmitic acid oxidised. On the whole we think that the amounts formed do not support the view that molecules of succinic acid were successively split off as groups of four carbon atoms from the palmitic chain after oxidation at the γ- or δ-carbon atom [*cf.* Clutterbuck, 1927] but rather that the four terminal carbon atoms had been oxidised to succinic acid.

Volatile acids of low molecular weight were formed including propionic, acetic and formic acids and lactones containing from four to seven carbon atoms; propaldehyde and acetaldehyde were identified and propionic acid as an oxidation product of the volatile lactones. The results obtained appeared to support the view that volatile fatty acids of low molecular weight and their hydroxy-derivatives were split off from the long carbon chain.

Whatever may be the mechanism involved, oxidation by means of hydrogen dioxide in the presence of a cupric salt through intermediate stages of desaturation and hydroxylation certainly appears to be the most effective laboratory process by which the higher fatty acids may be broken down to carbonic acid and the lower fatty acids, and suggests that some such process may be involved in oxidation *in vivo*.

Method of experiment.

2 g. palmitic acid were heated at 90° with the calculated amount of *N* NaOH solution and with 15 ml. of a 2·73 % solution of crystallised copper sulphate, 212 ml. water and such an amount of *N* NaOH as was found necessary to neutralise the amount of 20 vol. H_2O_2 solution to be added gradually during the experiment. The flask was fitted with a dropping funnel and with a reflux condenser from which a tube led to a series of flasks containing *N*/2 baryta solution. At the end of the specified time, an amount of *N* sulphuric acid exactly equivalent to the *N* NaOH originally taken was added and the flask cooled,

whilst air freed from carbon dioxide was drawn through the apparatus. After filtering off any insoluble residue a measured quantity of *N* alkali was added to destroy the dioxide, and when this had been accomplished, the equivalent quantity of *N* H_2SO_4 was added, the liquid concentrated either by steam-distillation or *in vacuo* and the residual liquid continuously extracted with ether. The distillate was neutralised with *N* NaOH, the first part of the neutral distillate collected and examined for neutral substances and the residue taken to dryness. The average molecular weight of the volatile acids was calculated from the amount of *N* NaOH solution used for neutralisation and the weight of dry sodium salts.

Experimental results.

In a series of preliminary experiments, the reaction mixture was heated at 60° for $3\frac{1}{2}$ hours with a large excess of hydrogen dioxide: about 80 % of the original palmitic acid was recovered unchanged; the 20 % which had disappeared had been converted into carbonic acid (8 % total carbon), volatile acids (8 %) and the remainder into a mixture of ether-soluble substances from which succinic acid was isolated. There were indications of the presence of an acid of high molecular weight giving a crystalline dinitrophenylhydrazone of unsharp melting-point and also of reducing substances. A water-soluble acid was present giving the iodoform reaction in the cold. There was however no evidence of the presence of methyltridecylketone such as would have been expected if β-keto-oxidation

Table I.

2 g. palmitic acid heated with H_2O_2 and Cu salt at 90°.

	Minutes heated	ml. 6 % H_2O_2	Wt. Cu palmitate and palmitic acid g.	Total wt. CO_2 g.	Wt. Na salt of volatile acids g.	Av. mol. wt. of volatile acids g.	% volatile reducing acid calculated as formic*	Total wt. of ether extract g.	Wt. of succinic acid g.
			Volatile acids separated by steam-distillation.						
A.	11	50	1·29	0·37	0·28	75	10·1	0·44	Negative
	20	75	1·34	0·37	0·28	75	10·1	0·44	Negative
B.	20	110	0·40	0·42	0·90	84	12·75	1·25	Positive
	15	80	0·23	0·47	0·85	78	12·29	>0·55	Positive
C.	60	175	0·21	0·68	1·02	74	18·6	1·6	0·1
	60	175	0·04	0·89	1·11	74	18·3	1·22	0·1
	60	200	0·30	0·79	1·08	63	13·15	1·21	—
	60	200	—	1·21	1·31	56	15·3	1·34	—
			Volatile acids separated by concentration *in vacuo*.						
D.	60†	175	0·16	—	0·33	67	23·8	{0·1 neutral 0·8 acid}	0·1
	60	175	0·29	—	0·52	68	34·0	1·2	0·1
	60†	175	0·30	—	0·47	—	33·0	1·0	0·05
	60†	175	0·32	—	0·60	73	—	0·81	0·11
	60	175	0·19	—	—	51	24·3	—	—
	60†	175	0·25	—	1·25	131	10·7	0·90	>0·1
	60	175	0·15	0·72	0·43	64	—	—	—
			Volatile acids separated by steam-distillation.						
E.	120	185	0·40	1·49	1·20	64	14·0	1·2	—
	120	285	—	1·49	1·20	54	5·6	—	—
F.	240	285	0·10	2·81	1·30	117	3·5	0·30	—
	240	285	0·0	2·9	1·08	53	3·8	0·17	—

* The reducing power was estimated by the reduction of mercuric chloride to calomel and calculated for purposes of comparison, but since formic acid is largely converted into CO_2 under the conditions of the experiment, other volatile reducing acids may have been present.

† In each of these experiments larger quantities of palmitic acid were oxidised and the amount of product calculated as derived from 2 g. of palmitic acid.

had taken place, as is known in the absence of the cupric catalyst. The further experiments were carried out at 90° at which temperature practically all the palmitic acid was oxidised.

After heating for 10–20 minutes at 90° with a limited amount of hydrogen dioxide, carbonic acid was evolved, and if the experiment was stopped while a considerable amount of the bluish insoluble precipitate which had separated remained, about 2/3 of the original palmitic acid was recovered, part of it as copper palmitate. About 7 % of the total carbon had been converted into carbonic acid, about 5 % into volatile acids and the remaining 8 % was present in the ethereal extract. The residue from the latter was neutralised and the solution extracted with ether to remove any neutral substances. The oil obtained from this neutral fraction contained 66·78 % C and 10·14 % H. It appeared to be a mixture of lactones corresponding approximately with the formula $C_8H_{14}O_2$ which requires C, 67·61; H, 9·86 %.

The behaviour on titration of the oil obtained from the acid fraction after it had been dried to constant weight in a vacuum-desiccator suggested the presence of a lactonic acid, since the colour of the indicator repeatedly disappeared on warming with additional quantities of $N/10$ NaOH. (Found for the free acid (micro-analysis) C, 64·13; H, 9·64 %. Mol. wt. by titration, 224 to 260; from analysis of Ag salt, 244. $C_{13}H_{24}O_4$ requires C, 63·93; H, 9·84 %. Mol. wt. 244.) Attempts to prepare a crystalline derivative from this acid were unsuccessful, and no evidence of the presence of the keto-group was obtained. A reducing group was present, since when warmed for a considerable time with ammoniacal silver nitrate solution a good silver mirror was deposited.

When the acid was warmed for one hour on a boiling water-bath with 50 % sulphuric acid, a strong gorse-like smell, characteristic of fatty lactones was developed, and a very small amount of a neutral substance containing 70·40 % C and 10·40 % H was isolated agreeing with the theoretical values for the lactone $C_{10}H_{18}O_2$ (70·59 % C; 10·59 % H).

From its behaviour on titration it appeared that the fraction having the composition $C_{13}H_{24}O_4$ contained a certain percentage of lactone but that most of it consisted of free acid, the proportions of carbon and hydrogen agreeing with those required by a ketohydroxy-acid. Since however the keto-group was not detected and could certainly not have been there to any large extent, it seemed probable that the bulk of this product was an unsaturated dihydroxy-acid. The substance reduced permanganate in neutral solution. At this stage no succinic acid was detected.

The formation of a product agreeing with the composition $C_{13}H_{24}O_4$ makes it probable that not only had γ- or δ-hydroxylation of the original C_{16} acid taken place but that hydroxylation had also taken place at two or three other points of the chain. The unsaturated linkage present in the C_{13} product may indicate that hydroxylation of the atom in the β-position to the 13th carbon atom had occurred with subsequent elimination of water, and the presence of a lactone that either the γ- or δ-carbon atom had been hydroxylated. Since, after heating with sulphuric acid, the lactone isolated had the composition $C_{10}H_{18}O_2$, it is possible that the remaining OH-group was in the γ- or δ-position to the 13th carbon atom.

When the proportion of hydrogen dioxide was increased, a point was reached at which rapid solution of nearly all the precipitate floating on the surface of the reaction mixture occurred, and practically the whole of the palmitic acid had been converted into water-soluble products. At this stage the quantity of carbonic acid given off was only slightly increased, but the amount of volatile

acids separating on steam-distillation was very much greater, and the average molecular weight showed that acids of higher molecular weight than acetic acid were present (*cf.* Table I). The residue from the ether extract was much increased, and its behaviour on titration again showed that a lactonic acid was present. Crystals of succinic acid were isolated and identified by a mixed melting-point.

When the reaction product was heated with a considerable excess of hydrogen dioxide for one hour, the quantities of carbonic and volatile acids produced were increased, but the average molecular weight of the latter was diminished. When the product was concentrated by distillation *in vacuo*, the total weight of volatile acids was less than half of that obtained when the volatile acids were separated by steam-distillation, and the proportion of a reducing volatile acid was much higher. The ether extract of the product obtained after the residue had been concentrated *in vacuo* smelt of acetic acid and only reached constant weight after long evacuation in a vacuum desiccator. The difference shown in the amount of volatile acids obtained by the two different methods of working up (*cf.* Table I) may be reckoned as due to the incomplete separation of the volatile acids, since during the concentration *in vacuo*, the product was not taken to complete dryness. In one experiment, the volatile distillate was neutralised with NaOH solution and extracted with ether. (Found (micro-analysis) C, 54·71; H, 8·97 %. $C_6H_{12}O_3$ requires C, 54·54; H, 9·09 %.) The amount was very small and no evidence as to structure was obtained.

In another experiment the sodium salts of the volatile acids were treated with strong H_2SO_4, and the diluted product was extracted with ether. The ethereal layer was shaken with potassium carbonate solution and the alkaline aqueous layer acidified and extracted with ether. From this ethereal solution an oil was obtained. (Found C, 43·99; H, 1·63 %. Mol. wt., by titration, 108; by analysis of Ag salt, 95. $C_4H_8O_3$ requires C, 46·15; H, 1·69 %. Mol. wt. 104.) From the neutral ethereal layer separated from the potassium carbonate solution, a substance of characteristic lactone odour was separated. (Found C, 64·53; H, 9·81 %. $C_6H_{10}O_2$ requires C, 63·16; H, 8·77 %. $C_7H_{12}O_2$ requires C, 65·63; H, 9·38 %.) Volatile hydroxy-acids and lactones had therefore been formed as decomposition products.

In the neutral volatile portion (obtained by redistilling the neutralised distillate, which had been separated either by steam-distillation or by concentration under diminished pressure), propaldehyde was identified by means of its orange-red dinitrophenylhydrazone, melting after recrystallisation from alcohol at 153–154° and the result was confirmed by analysis of the hydrazone. (Found N, 24·05 %. $C_9H_8N_4O_4$ requires N, 23·73 %.)

After oxidising the volatile acids with chromic acid, a neutral product giving analytical figures between those required for the lactones $C_6H_{10}O_3$ and $C_7H_{12}O_3$ was obtained. When the sodium salts of the volatile acids were treated with strong sulphuric acid and the product distilled, propionic acid was identified in the distillate and separated as the characteristic basic lead salt. The routine method for estimating formic acid by reduction of mercuric chloride was also applied to the mixture of volatile acids and very considerable reduction took place. Since however formic acid is readily destroyed under the conditions of the experiment, some other reducing volatile acid may have been present.

After one hour's heating, for every 2 g. of palmitic acid oxidised, between 0·1 and 0·2 g. succinic acid was isolated as crystals from the mixture, but there was evidence that considerably more was formed.

The residue from the ethereal extract was divided into two parts by means

of its solubility in benzene. In one experiment in which 24 g. of palmitic acid had been oxidised, 6·72 g. of an oil insoluble in benzene and 4·6 g. of one soluble in benzene were obtained.

From the oil insoluble in benzene more than 1 g. of succinic acid separated as crystals and the residue was divided into two fractions: (1) a part soluble in water of low average molecular weight (94 by silver salt analysis, 122 by titration) containing a considerable amount of succinic acid and giving a silver salt which rapidly darkened; (2) a part insoluble in water. The latter had an average molecular weight of 167, determined by titration and apparently contained about 20 % lactone. (Found C, 53·74; H, 7·87 %.) Its iodine value was 22·4. The simplest formula agreed most nearly with $C_7H_{12}O_4$, which requires C, 52·50; H, 7·50 %. Since the proportion of carbonyl group determined by its reaction with sulphite was negligible, and considering the proportion of carbon to hydrogen atoms, unsaturated hydroxy-acids must have been present with a small proportion of hydroxylactone.

The composition of the water-soluble fraction (1) varied in different experiments from C, 52·7 to 56·5; H, 7·1 to 7·5 %, and it contained an appreciable amount of succinic acid. Estimation of the carbonyl group showed that less than 2 % was present; the iodine value was 35, indicating a considerable proportion of unsaturated acid in the mixture.

The composition of the fraction soluble in benzene varied in a number of different experiments from 58·44 to 65·69 % C and from 7·8 to 9·7 % H. The molecular weight determined by titration gave values from 150 to 200 and the product contained about 21 % lactone. The product was divided into neutral and acid fractions.

The neutral product appeared to consist of a mixture of hydroxylactones for the separation of which neither distillation nor fractionation by means of solvents has yet given satisfactory results. A fraction insoluble in light petroleum contained 63·00 % C and 8·08 % H. Its molecular weight (determined by heating a known weight for one hour on a water-bath dissolved in alcohol with a known amount of NaOH and titrating the excess) gave a molecular weight of 174, but the end-point was not very satisfactory. This substance did not react with a solution of sodium bisulphite and was therefore free from any appreciable amount of keto-compound. Its iodine value was 68, corresponding with the existence of rather less than half an ethylene linkage for the observed molecular weight. As already pointed out an ethylene linkage in the $\alpha\beta$-position gives a very low iodine value. The available evidence would point to the presence of the lactone of an unsaturated hydroxy-acid corresponding approximately with the formula $C_9H_{14}O_3$, which requires C, 63·53; H, 8·23 %. Mol. wt. 170. Until a better method of separation and identification has been found a definite formula cannot be ascribed to it.

The acid fraction was converted into sodium salts, the solution of which was evaporated to dryness and the dry salts fractionated by their solubility in 96 % alcohol and acetone. The acid derived from a very hygroscopic sodium salt soluble in alcohol and not precipitated from alcoholic solution by the addition of acetone agreed in composition with the formula $C_{10}H_{18}O_4$. (Found C, 59·58; H, 8·39 %. Mol. wt. by titration 200, 203. $C_{10}H_{18}O_4$ requires C, 59·40; H, 8·91 %. Mol. wt. 202.) It no longer reacted as a lactonic acid, and the estimation of the carbonyl group was negative. The I.V. was 16·5. It may therefore be regarded as a dihydroxydecenoic acid.

For purposes of reference we synthesised the α-keto-derivatives of octanoic, nonanoic and decanoic acids and examined their properties. The method adopted

was that used by Bouveault and Locquin [1904], in which nitrosylsulphuric acid was allowed to act upon the ethyl ester of the α-acetyl derivative of the normal fatty acid in the presence of concentrated sulphuric acid, when the acetyl group was replaced by the group —NOH. By the further action of nitrosylsulphuric acid in the presence of formic acid the corresponding α-keto-acid was obtained.

Preparation and properties of α-keto-octanoic acid.

The ethyl ester of α-acetyloctanoic acid was prepared by heating together equimolecular proportions of sodium ethoxide, ethyl acetoacetate and hexyl iodide. The oxime of this ester (M.P. 52–53°) was prepared and, after recrystallisation, converted into α-keto-octanoic acid [Bouveault and Locquin, 1904; Blaise and Luttringer, 1905]. This was a colourless crystalline substance, smelling strongly of "goat," which melted at 117–118° after recrystallisation from alcohol and subsequent washing with light petroleum, in which it was sparingly soluble. It formed an oxime, M.P. 110°, and a bright yellow dinitrophenylhydrazone which, after two recrystallisations from alcohol, melted at 136·5–137·5°. The acid gave a silver mirror on warming with ammoniacal silver nitrate. (Found: C, 60·28; H, 8·76 %. Theoretical: C, 60·76; H, 8·86 %.)

Preparation and properties of α-ketononanoic and α-ketodecanoic acids.

These acids were prepared in a similar manner. α-Ketononanoic acid was derived from the ethyl ester of α-acetylnonanoic acid [Jourdan, 1880]; the oxime was a white crystalline substance melting at 58–59° after recrystallisation from dilute acetic acid. α-Ketononanoic acid melted at 105–106°, formed an oxime melting at 112°, and a dinitrophenylhydrazone melting at 129°. It reduced ammoniacal silver nitrate. (Found: C, 62·26; H, 9·08 %. Theoretical: C, 62·78; H, 9·30 %.) α-Ketodecanoic acid was prepared from ethyl α-acetyldecanoate [Guthzeit, 1880]; the oxime melted at 60–61°. The α-keto-acid melted at 85–86° after recrystallisation from light petroleum. It gave a dinitrophenylhydrazone, M.P. 126–128°, and a silver mirror with ammoniacal silver nitrate. (Found: C, 64·16; H, 10·15 %. Theoretical: C, 64·51; H, 9·68 %.)

SUMMARY.

Although we have not yet been able to characterise substances of undoubted chemical individuality from the complex mixture of ether-soluble products obtained on oxidising palmitic acid by means of hydrogen dioxide in the presence of a cupric salt, certain inferences as to the nature of the products and therefore as to the intermediate stages of the process can be made.

1. Hydroxylation appeared to take place simultaneously at several points of the palmitic chain.

2. The palmitic carbon chain was gradually broken down, and evidence of the formation of unsaturated hydroxy-acids containing ten carbon atoms was obtained. There was also some indication that chains containing respectively thirteen and seven carbon atoms were formed during the reaction, but the chemical individuality of these substances requires further confirmation.

3. Ketonic acids were present, if at all, only in very small quantities and were certainly not the main product of oxidation.

4. Lactones, indicating the formation of γ- or δ-hydroxy-acids were split off during the course of the reaction.

5. Unsaturated hydroxy-acids were present and, from the analyses of the products, these formed the main bulk of the intermediate substances. The low

iodine values recorded probably indicated the presence of some unsaturation in the 1:2-position.

6. Volatile acids of low molecular weight were formed in considerable quantity and were split off at an early stage.

7. When heated for 4 hours at 90° more than 50 % of the total carbon of the palmitic acid appeared as CO_2 and more than 20 % as volatile acids.

8. For purposes of reference the α-ketoheptanoic and α-keto-octanoic acids were prepared and characterised.

The study of these intermediate compounds is in progress and the result will form the subject of a further communication.

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REFERENCES.

Battie and Smedley-MacLean (1929). *Biochem. J.* **23**, 593.
Blaise and Luttringer (1905). *Bull. Soc. Chim.* **33**, 827.
Bouveault and Locquin (1904). *Bull. Soc. Chim.* **31**, 591, 1058, 1142.
Clutterbuck (1927). *Biochem. J.* **21**, 512.
—— and Raper (1925). *Biochem. J.* **19**, 385.
Dakin (1908, 1). *J. Biol. Chem.* **4**, 221.
—— (1908, 2). *J. Biol. Chem.* **5**, 173, 303.
—— (1909). *J. Biol. Chem.* **6**, 203.
Gröger (1887). *Monatsh. Chem.* **8**, 490, 497.
Guthzeit (1880). *Liebig's Ann.* **204**, 2.
Jourdan (1880). *Liebig's Ann.* **200**, 105.
Nördlinger (1886). *Ber. deutsch. chem. Ges.* **19**, 1899.
Traube (1905). *Ber. deutsch. chem. Ges.* **38**, 830.
—— (1906). *Ber. deutsch. chem. Ges.* **39**, 178.
—— (1910). *Ber. deutsch. chem. Ges.* **43**, 763.
—— (1911). *Ber. deutsch. chem. Ges.* **44**, 3141.
—— (1932). *Ber. deutsch. chem. Ges.* **65**, 187, 190.
Smedley-MacLean and Pearce (1931). *Biochem. J.* **25**, 1252.
—— and Thomas (1921). *Biochem. J.* **15**, 319.

LXXI. NOTE ON THE STORAGE OF CARBOHYDRATE AND FAT BY *SACCHAROMYCES FROHBERG* WHEN INCUBATED IN SUGAR SOLUTIONS.

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THE storage of carbohydrate and fat by yeast on incubation in various sugar solutions (glucose, fructose, sucrose, maltose, lactose) was studied by Smedley-MacLean and Hoffert [1923; 1924]; the investigation has now been extended to include galactose.

The brewery yeast used in the previous experiments, a mild ale yeast which did not ferment galactose, produced on incubation in a 4 % solution of this sugar no increase in fat and a fall in carbohydrate. *Saccharomyces Frohberg*, a yeast known to ferment galactose after acclimatisation, was therefore tried. This yeast was acclimatised to galactose by culturing on a hydrolysed lactose and yeast extract medium, and grown, at laboratory temperature, on a 5 % sucrose and yeast extract medium, 4 litres of which furnished about 20 g. of yeast after a week's growth.

6·25 g. of the *Frohberg* yeast were analysed as a control, and two portions of 6·25 g. incubated in 500 ml. of each of the two solutions to be compared, for 44 hours, with oxygenation, at room temperature (*ca.* 15°).

The methods of estimation of carbohydrate and fat were those described in the previous communication.

Possibly owing to the different media on which they had been grown, the carbohydrate content of the *Frohberg* was somewhat lower than that of the brewery yeast, while the fat content of the former was nearly twice as great.

The storage in 2, 4 and 5 % glucose and galactose solutions was studied, equal portions of the same sample of yeast being incubated, under the same conditions, in the two solutions to be compared. An increase of carbohydrate storage and of fat storage with increasing concentration of sugar was shown both in glucose and galactose solutions. There was no significant difference between the amount of carbohydrate stored on incubation in the same concentration of glucose or galactose; in glucose, however, more fat was stored at all concentrations than in galactose (see Figs. 1 and 2).

The effect of the presence of phosphate (0·49 g. $Na_2HPO_4, 12H_2O + 0·143$ g. KH_2PO_4 per 500 ml. of solution, p_H about 7·8) in 5 % solutions of glucose and galactose was then observed. It was shown previously that addition of phosphate to solutions of glucose, fructose, sucrose and maltose, brought about an increased storage of fat and also of phosphorus in brewery yeast incubated therein. This effect was marked and led to the suggestion that fat formation in yeast proceeded *via* hexosephosphate. The *Frohberg* yeast behaved differently. When it was incubated in a 5 % glucose solution to which phosphate had been

added there was no increase in fat storage, the variation was rather a decrease but not of significant amount; the presence of phosphate in a 5 % galactose solution caused a slight increase in fat, but this was not of the same order as the increases referred to above.

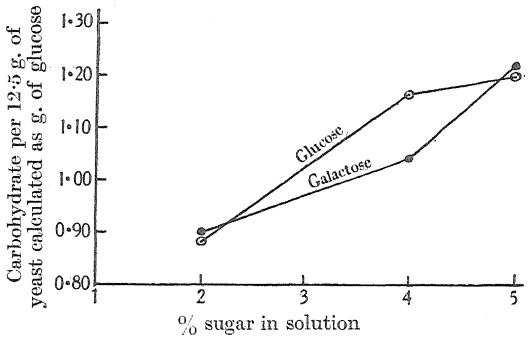

Fig. 1.

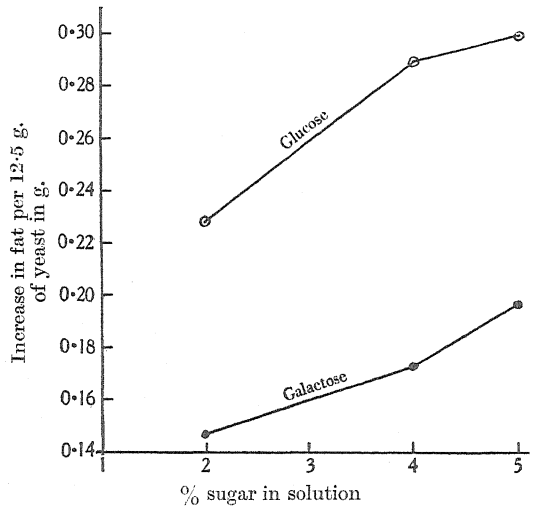

Fig. 2.

Estimation of phosphorus content, by Neumann's method, showed no significant variations.

A somewhat more marked effect seems to be shown on carbohydrate storage. The addition of phosphate to a glucose solution caused a diminution, to a galactose solution an increase, in carbohydrate content.

Estimation of the residual sugar in a solution in which yeast had been incubated, showed that in parallel experiments, nearly twice as much glucose was used up as galactose. Tests were performed to ascertain whether the *Frohberg* yeast lost its power of fermenting galactose when grown on sucrose media and the results indicated that this was so; since the amount of galactose (up to 10 g.) used up during a 44-hour incubation could not, however, have been accounted for by increased storage within the cell and some fermentation of galactose must have taken place though this was probably retarded. There was no

Table I.

The numbers represent g. per 12·5 g.* original yeast.

% sugar in medium		Original yeast		After incubation in glucose solution		After incubation in glucose + phosphate solution		After incubation in galactose solution		After incubation in galactose + phosphate solution		After incubation in maltose solution		After incubation in fructose solution		After incubation in sucrose solution	
		Carbo-hydrate g.	Fat g.	Carbo-hydrate g.	Fat g.	Carbo-hydrate g.	Fat g.	Carbo-hydrate g.	Fat g.	Carbo-hydrate g.	Fat g.	Carbo-hydrate g.	Fat g.	Carbo-hydrate g.	Fat g.	Carbo-hydrate g.	Fat g.
2		0·72	0·172	1·00	0·406	—	—	0·88	0·288	—	—	—	—	—	—	—	—
		0·82	0·166	0·76	0·414	—	—	0·88	0·314	—	—	—	—	—	—	—	—
		1·00	0·144	0·84	0·374	—	—	0·96	0·330	—	—	—	—	—	—	—	—
		0·98	0·128	0·92	0·376	—	—	—	—	—	—	—	—	—	—	—	—
	Mean	0·88	0·152	0·88	0·392	—	—	0·90	0·310	—	—	—	—	—	—	—	—
4		0·70	0·178	1·10	0·496	—	—	1·03	0·364	—	—	—	—	—	—	—	—
		0·63	0·156	1·22	0·410	—	—	1·07	0·310	—	—	—	—	—	—	—	—
		—	—	1·13	0·450	—	—	—	—	—	—	—	—	—	—	—	—
	Mean	0·66	0·166	1·16	0·454	—	—	1·04	0·336	—	—	—	—	—	—	—	—
5		0·96	0·238	1·41	0·552	0·96	0·386	1·46	0·406	1·74	0·508	0·90	0·250	1·16	0·380	1·02	0·370
		1·06	0·150	1·00	0·492	0·78	0·482	1·14	0·366	0·88	0·338	1·33	0·294	0·98	0·346	1·24	0·348
		0·93	0·182	1·12	0·482	0·90	0·366	1·34	0·370	1·20	0·404	—	—	—	—	—	—
		0·84	0·166	1·27	0·366	0·82	0·368	1·08	0·338	1·50	0·348	—	—	—	—	—	—
		0·82	0·158	—	—	—	—	1·05	0·316	—	—	—	—	—	—	—	—
		0·74	0·158	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		0·94	0·180	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		0·96	0·194	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		0·94	0·180	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		1·02	0·152	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		0·67	0·158	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		0·71	0·138	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Mean	0·84	0·160	1·20	0·464	0·86	0·400	1·22	0·360	1·32	0·400	1·11	0·272	1·07	0·363	1·13	0·359

Milligrams phosphorus per 12·5 g. of original yeast in g.

		Mean
Original yeast	72, 66, 68, 76, 98	76
After incubation in 5 % glucose solution	74, 44 — — —	60
After incubation in 5 % glucose + phosphate solution	64, 98, 88, 106 —	88
After incubation in 5 % galactose solution	80, 56, 84 — —	74
After incubation in 5 % galactose + phosphate solution	84, 70, 66, 54 —	68

* Results are given per 12·5 g. of yeast for comparison with previous work on brewery yeast.

difference between the amount of sugar used up from a sugar solution and from a similar solution with phosphate.

The *Frohberg* yeast was also incubated in 5 % solutions of maltose, fructose, and sucrose. The carbohydrate storage, in these sugars, was of the same order as in glucose and galactose at the same concentration; particularly remarkable was the total absence of the extra carbohydrate storage shown by brewery yeast in maltose, attributed then to the possible direct formation of glycogen from maltose. Fat storage was poorer in maltose solution than in galactose and in fructose and sucrose of the same order as in galactose; it was in no case so good as in glucose.

SUMMARY.

1. *Carbohydrate storage.* The carbohydrate storage of *Saccharomyces Frohberg* on incubation in solutions of glucose, galactose, fructose, sucrose and maltose of the same strength is similar in all cases. Glucose and galactose were investigated in varying concentration whereby it was shown that there was an increased storage of carbohydrate with increased concentration of either sugar in the medium.

Frohberg yeast behaved similarly to brewery yeast when incubated in solutions of glucose, galactose, fructose and sucrose but showed a marked difference on incubation in maltose. While *Frohberg* yeast stored normal amounts of carbohydrate in the case of this sugar, in brewery yeast an abnormally high carbohydrate storage occurred.

2. *Fat storage. Frohberg* yeast showed a greater power of forming fat when incubated in solutions of glucose than in those of galactose, fructose, sucrose, or maltose; the last seemed the least effective. In the cases of glucose and galactose the amount of fat stored increased with increase of sugar in the surrounding medium. The addition of phosphate to the medium did not appear to cause any such increase of fat storage as was previously noted in the case of brewery yeast.

REFERENCES.

Smedley-MacLean and Hoffert (1923). *Biochem. J.* **17**, 720.
—— —— (1924). *Biochem. J.* **18**, 1273.

LXXII. THE OSMOTIC PRESSURE OF GUM ACACIA SOLUTIONS.

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(*Received February 13th, 1934.*)

The great and increasing use of solutions of gum acacia, with or without the addition of sodium chloride, as an infusion in cases of shock and loss of blood [Bayliss, 1918], together with the mass of data now available relating to the osmotic pressure of the blood-colloids in health and disease, render an exact knowledge of the osmotic pressure of the colloids in gum acacia solutions of importance.

The osmotic pressure of the solution is likely to be modified by such factors as the concentration of salts present, the heat treatment to which the solution has been submitted for purposes of sterilisation and, perhaps, the dilution of the gum, a concentrated solution containing larger complexes than one more dilute.

It was, therefore, decided to study the effects of concentration, of the addition of salts and of the influence, if any, of different methods of sterilisation upon the osmotic pressure of the colloids in solutions of gum acacia.

Methods.

Apparatus. For the majority of the experiments recorded below the Krogh [Krogh and Nakazawa, 1927] type of osmometer, made by the Zsigmondy Ultrafilter company, was used. In a few of them the osmometer used by Widdowson [1933] was employed. The osmometers were set up in all the three possible positions—dialysate above plate with bent capillary; dialysate below plate (Krogh); dialysate above plate with straight capillary—and, as concordant results were obtained with all of these, the pressures measured were obviously real. A loose-fitting cap containing damp cotton wool covered the open ends of the osmometers in cases one and three above, while the end dipped into water when in the "Krogh" position. Evaporation of the dialysate was thus prevented. Pressures were recorded in cm. of water.

Membranes. In the present series of experiments only cellophane ("No. 600") as used by Widdowson and by Govaerts [1927] was employed.

Time and temperature. Between 22 and 24 hours was allowed for equilibrium to be attained. 17 hours was definitely too short a time and in several cases good readings were obtained after a lapse of 46 hours. One of the osmometers was accommodated in a vacuum flask, but this did not seem to add much to the consistency of the readings. All pressures were reduced to 0°.

Material. Finest commercial "Gum acacia parv" was used for making the test solutions. Solutions were never more than 3 days old for "fresh" gum.

When sterilised material was employed it was sterilised in small bottles and a fresh one used if the previous one had been open for more than 3 days. Some fresh

gum solutions were preserved by the addition of sufficient thymol to saturate the mixture.

The standard infusion solution used clinically contains 6 % of gum acacia. This strength was made a standard and all results referred to it.

Effect of concentration on osmotic pressure.

Solutions of varying strength were examined. It was not possible, with the apparatus in use, to measure pressures much higher than were given by a solution of 6 % concentration.

Table I.

Strength of solution %	Osmotic pressure observed cm. water at 0°	Osmotic pressure for 6 %
2	28·1	84·3
2	23·9	71·7
3	37·4	74·8
3	40·4	80·8
3	40·0	80·0
3	38·8	77·2
6	80·8	80·8
6	77·7	77·7

Mean value (for 6 % gum) 78·4 cm. p_H 4·4–4·5.

The results in Table I show that the osmotic pressure is not affected within the limits of experimental error by alteration in the strength of the solution between the limits 2–6 %. For solutions saturated with thymol the results are given in Table II.

Table II.

Strength of solution %	Osmotic pressure observed cm. water	Osmotic pressure for 6 % at 0°
2	23·82	71·46
2	25·55	76·65
3	40·1	80·2
3	36·5	73·0
4	48·9	73·3
6	73·4	73·4

Mean value (for 6 % gum) 74·7 cm.

Again it is obvious that dilution has not affected the observed osmotic pressure. The presence of thymol causes a diminution of 4·9 % in the osmotic pressure.

Effect of the addition of sodium chloride (Table III).

Solutions containing 6 % of gum and 0·9 % salt (corresponding to the clinical solution of 6 % gum in normal saline) were examined.

Mean osmotic pressure observed 11·93 cm.; p_H 4·2.

Table III. *Effect of different concentrations of sodium chloride.*

Gum 6 % containing salt (%)	Mean osmotic pressure	p_H
2·0	11·0	—
0·9	11·93	4·2
0·46	15·8	4·3
0·31	18·0	—
0·276	18·26	4·4
0·2	21·6	—
0·1	32·6	—
0·05	49·5	—
Nil	78·4	4·5

The addition of salt has therefore a profound depressing action on the osmotic pressure of the colloids in gum solutions.

6 % gum in 0·9 % saline saturated with thymol, 11·32 cm.

Saturating the solution with thymol depresses the osmotic pressure by the same proportion (4·9 %) as was found for pure gum solutions.

The figures obtained all lie on a smooth curve of hyperbolic type, and no discontinuity is observed. (See Fig. 1.)

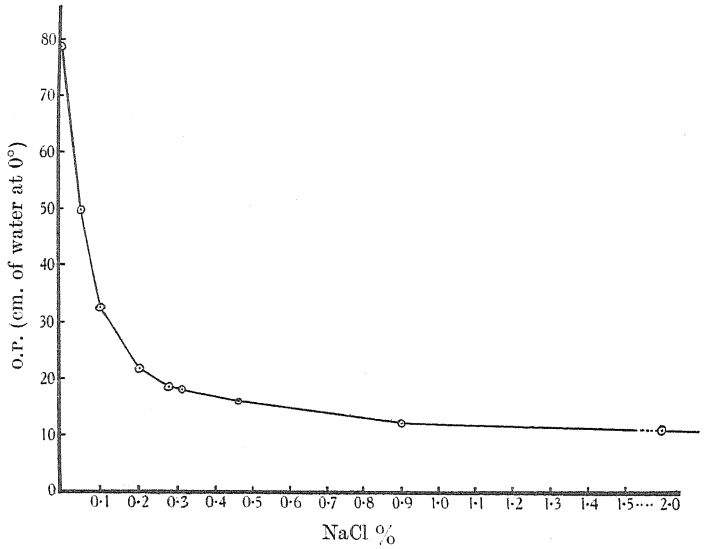

Fig. 1. Osmotic pressure of 6 % gum acacia solution with varying amounts of NaCl.

The p_H of the solution falls proportionately as the salt content rises. This is due to a change in the gum, not to the salt itself, since 3 % gum in normal saline has a p_H of 4·3 as against the value of 4·2 for 6 % gum containing the same amount of salt.

Effect of dialysis (Table IV).

In view of this series of results it was decided to measure the osmotic pressure of gum solutions purified from salts, naturally present in the solution, by dialysis.

Pure gum solutions were dialysed in a pair of Asheshov's tubes, fitted with a cellophane membrane, against distilled water. The gum was preserved by the addition of thymol as above and the distilled water was also saturated with this preservative. The osmotic pressures were corrected for the depressing action of thymol by the amount found to be correct from earlier work. The water was changed daily.

Table IV. *Effect of dialysis.*

	Osmotic pressure cm.
Gum solution 1 month old dialysed for 5 days gave	83·6 for 6 %
Gum dissolved in apparatus and dialysed 7 days gave	115 for 6 %
Gum freshly dissolved	78·4

The difference between the two results above may be ascribed to one of three causes or to a combination of them.

1. Thymol does not protect the gum completely against bacterial action.
2. The osmotic pressure of a gum solution may alter naturally with time.
3. The second solution was dialysed for a longer time.

It is considered that the first cause cited above is the most likely.

As expected, purification of the gum solution by dialysis caused a rise in the observed osmotic pressure.

Sterilisation.

The effect of different types of heat treatment on the osmotic pressure was investigated on four solutions containing 6 % of gum. The treatments to which these were subjected were as follows (Table V).

Table V. *Effect of heat treatment.*

Solution A, steamed for 20 minutes on 3 successive days; B, autoclaved to 30 lbs. pressure; C, autoclaved to 30 lbs. pressure and then to 7 lbs. for 10 mins.; D autoclaved to 30 lbs. pressure, and then steamed on 3 successive days.

The osmotic pressures are those for 6 % concentration.

Solution	Mean osmotic pressure observed cm. water	p_H
A	80·9	4·55
B	77·3	4·65
C	98·2	4·55
D	101·6	4·60
Fresh gum	78·4	4·4–4·5

For gum solutions containing 0·9 % of sodium chloride the values in Table VI were obtained (the letters refer to the same heat treatment as in the pure gum series).

Table VI.

Solution	Mean osmotic pressure	p_H
A	12·87	4·4
B	12·59	—
C	15·91	4·3–4·2
D	17·04	4·3
Fresh gum saline	11·93	4·2

The conclusions to be drawn from these two sets of results are that, while sterilising by any method does break up the gum molecule, such damage is not so profound as to render the resulting particles dialysable to any marked extent. If this had occurred a diminution in the value of the osmotic pressure would have been found instead of the increase actually met with.

The second sterilisation (after filling into the final container) does more damage than the first and steaming on 3 successive days is more active as a disruptive agent than is the autoclave treatment.

The same order of increase in osmotic pressure is noted in both sets of solutions, but the proportionate increase is greater when salt is present (Table VII).

Table VII

Solution	Saline osmotic pressure	Pure (calc. from saline figures) osmotic pressure	Pure (observed) osmotic pressure
Fresh	11·98	(78·4)	(78·4)
A	12·87	85·3	80·9
B	12·59	83·5	77·3
C	15·91	105·5	89·2
D	17·04	112	101·6

The p_H of gum solutions is not markedly affected by sterilisation.

Discussion and summary.

The results detailed above show that while the colloid osmotic pressure of gum acacia is independent of the concentration over the range 2–6 %, it is greatly reduced by the presence of salt. The actual osmotic pressure of 6 % gum in normal saline is only little more than one-third of that of the blood-colloids, and, while sterilisation causes a considerable increase in the osmotic pressure, the latter is even then less than one-half that of the colloids in normal blood. It would seem that the addition of salt to gum solutions is, partially at least, defeating the clinical object of the administration of such treatment.

The sterilised gum solutions used in the work were supplied by The Crookes Laboratories.

REFERENCES.

Bayliss (1918). Intravenous injection in wound shock. (Longmans, Green and Co., London.)
Govaerts (1927). *Bull. Acad. Roy. Ned. Belge* [5], **7**, 356.
Krogh and Nakazawa (1927). *Biochem. Z.* **18**, 241.
Widdowson (1933). *Biochem. J.* **27**, 1321.

LXXIII. THE CHEMISTRY OF MOULD TISSUE[1]. V. FRACTIONATION OF THE NITROGEN IN THE MYCELIUM OF *ASPERGILLUS FISCHERI*.

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In view of the enormous number of mould species and the high nitrogen content of the mycelium (4–10 %), it is surprising to find how meagre are the data on the nitrogenous constituents of such tissues. A review of the literature shows that only four or five species have been investigated and most of these only casually.

Small quantities of alkali-soluble proteins have been isolated by several investigators [Thomas and Moran, 1914; Takata, 1929, 2; Thomas, 1928; 1930; Clutterbuck *et al.*, 1932]. Such proteins have been uniformly low in nitrogen (12–13 %); some have been high in phosphorus (0·7 %); and some have given strong tests for carbohydrate. On the basis of such data Takata [1929, 1] suggested that a protein which he isolated might be regarded as a phosphoglycoprotein. Certain it is that the proteins of the mould pad are difficult to isolate and appear to be different in character from most of the proteins of plant and animal materials.

A number of amino-acids have been identified in the acid hydrolysate of the mycelium or proteins separated therefrom. Glycine, alanine, leucine, tyrosine, lysine, arginine, histidine, tryptophan, aspartic and glutamic acids have been reported by one or more investigators [Takata, 1929, 2; Clutterbuck *et al.*, 1932; Abderhalden and Rona, 1905; Vorbrodt, 1919; 1921]. As shown by Abderhalden and Rona [1905], the mould, *A. niger*, was able to synthesise the same amino-acids from either inorganic nitrogen (*e.g.* potassium nitrate) or organic nitrogen (*e.g.* glycine or glutamic acid).

Indirect evidence from feeding experiments with animals [Takata, 1929, 1; Skinner *et al.*, 1933] indicates either the absence of certain essential amino-acids or their presence in too low a proportion to bring about growth at an intake level of 15–18 % protein. Such feeding trials also indicate considerable variation in amino-acid content among different species of moulds and it may be even in the same species under different conditions of growth.

Besides amino-acids, xanthine, hypoxanthine, guanine, adenine, cytosine, glucosamine, choline, betaine and stachydrine have been reported [Takata, 1929, 2; Vorbrodt, 1921] among the hydrolysis products of *A. niger* and *A. oryzae*. Whether or not such compounds are found generally in mould mycelium cannot be decided on the basis of the data now available.

[1] This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

In view of the incompleteness of the data, it seemed desirable (*a*) to separate the total nitrogen of mould pads into various fractions according to their solubilities and (*b*) to determine the partition of nitrogen in each of these fractions.

Experimental.

The mould tissue used in the following experiments was a pure culture of *A. fischeri* grown in batches of 25 kg. on a glucose-inorganic salts medium. The details of the procedure used in growing the mould have been reported in a previous paper [Peterson *et al.*, 1933].

Preliminary experiments.

Several preliminary nitrogen extraction experiments were conducted. While the data from these experiments cannot be presented here because of lack of space, the following statements give the more important results obtained.

Mould tissue which had been killed by steaming, then dried and ground and subjected to long extraction with hot alcohol to remove fat and sterols [Pruess *et al.*, 1932] proved to be unsatisfactory for nitrogen studies. Tissue that had been thus treated absorbed water and formed a slimy, jelly-like mass, which was not filtrable. The extract remained turbid even after passage through a Sharples super-centrifuge. The nitrogen of the mycelium distributed itself among successive solvents as follows: water, 11·7 %; 2 % NaOH, 28·2 %; 5 % NaOH, 30·8 %; alkali-insoluble, 18·9 %.

Mould tissue which was killed by steaming at 99° for one hour, then dried for 48 hours at 65° and ground was more satisfactory in that it could be filtered by draining overnight from fluted filters. The solubility of the nitrogen in this material was as follows: water, 18·9 %; 2 % NaOH, 26·7 %; 5 % NaOH, 28·7 %; alkali-insoluble, 17·1 %.

The best results were obtained when perfectly fresh untreated mycelium was used. Aqueous extracts of this material filtered readily and gave clear filtrates. However, complete extraction of water-soluble substances was obtained only after extraction of the residue with alcohol-ether following a first water extraction. When this was done, an additional quantity of water-soluble nitrogen, almost equal to that contained in the first extract, was obtained. 10 % sodium chloride solution or 1 % hydrochloric acid solution extracted about the same percentage of the total nitrogen as did water alone.

Extractions of the water-insoluble residue with alkaline (KOH, $NaHCO_3$, Na_2CO_3, $Ba(OH)_2$) solutions of various strengths and under different conditions of time and temperature were compared. 1 % KOH solution at room temperature followed by 4 % KOH solution at boiling temperature seemed to give the most regular and informative results.

On the basis of the preliminary experiments the final extraction procedure given below was adopted.

Method of extraction.

Mould tissue fresh from the pans of the steriliser-incubator was rinsed with distilled water and immediately ground to a fine paste in a "Nixtamal" mill. The ground pad was allowed to fall into a dish containing chipped ice and was stirred into the ice. When the pad had all been ground, it was emptied into a jar of iced water and stirred vigorously for 15 minutes with an electric stirrer. It was then filtered with suction on a cloth filter and the filtrate poured back upon the filter-cake and refiltered until it came through clear. The filter-cake was given two

more 15-minute extractions with iced water, and all three filtrates were combined and comprise the "First water-extract."

The filter-cake from the water-extract was thoroughly stirred into a large excess of alcohol-ether (1 : 1), and allowed to stand overnight. The alcohol-ether was filtered off, and the filter-cake was suspended in ether and allowed to stand several days before filtering. The filtrates were combined; they constitute the "Lipin fraction."

The ether-extracted filter-cake was spread out in a thin layer and air-dried before a fan for 2 hours. The dry powder was then stirred with water for 15 minutes and filtered. The filter-cake was re-suspended in water and allowed to soak overnight under protection of toluene. It was then filtered, and the two filtrates combined formed the "Second water-extract."

The residue from the water extraction was stirred into an excess of 1 % KOH solution and allowed to stand at room temperature for 2 days. The suspension was filtered with suction on a cloth filter, the filter-cake washed twice by stirring with water and filtering, and the three filtrates were combined. This is designated as "Cold 1 % KOH extract."

The filter-cake was boiled with 4 % KOH solution for one hour, filtered and washed twice with water, and the filtrates were combined to form the "Hot 4 % KOH extract."

The residue from the hot alkaline extraction was washed with 50 % alcohol, 95 % alcohol and ether and then dried. This fraction is called "Residue."

The extraction procedure as described above has been carried out a number of times, and two typical analyses (on batches 28 and 31) are presented in Table I.

Table I. *Nitrogen distribution of* A. fischeri.

	Percentage distribution				Percentage composition of fractions				Free amino-nitrogen. Percentage of total nitrogen in each fraction
	Nitrogen Batch		Solids (ash-free basis) Batch		Nitrogen (ash-free basis) Batch		Ash Batch		
Fractions	28	31	28	31	28	31	28	31	Batch 31
First water-extract	17·2	16·8	19·8	15·6	4·4	6·7	13·4	11·6	37·5
Lipins	1·2	1·2	9·5	8·5	0·9	1·1	0·0	0·0	†
Second water-extract	13·6	11·7	17·0	14·8	4·1	4·9	1·4	1·8	25·0
Cold 1 % KOH extract	39·7	41·2	21·1	22·9	9·6	11·1	35·0*	33·6*	9·4
Hot 4 % KOH extract	13·0	14·3	13·7	15·2	4·9	5·8	64·4*	54·1*	14·0
Residue	12·2	13·8	26·0	27·2	2·3	3·1	2·9	2·4	†
Total recovered	96·9	99·0	107·1	104·2	—	—	—	—	—
Whole mould	100·0	100·0	100·0	100·0	5·1	6·2	3·0	2·9	†

* Including ash from alkali. † Not determined.

Nitrogen distribution.

As can be seen from Table I, 17·2 and 16·8 % of the total nitrogen of the mould pad of batches 28 and 31, respectively, were dissolved in the first water-extract. The nitrogen content of the extracted solids in batch 28 was slightly less (4·4 %) than that of the whole mould (5·1 %), whereas in batch 31 it was slightly more (6·7 % against 6·2 %).

The first water-extract was a yellowish green solution of p_H 4·6. It gave negative biuret, Millon and Hopkins-Cole tests and a positive xanthoproteic test. It gave no precipitate with trichloroacetic, acetic or hydrochloric acid, but gave a slimy brown precipitate when made alkaline with KOH, and a further precipitate upon the addition of baryta to the filtrate from the KOH precipitation. Neither of these two precipitates contained as much as 1 % of nitrogen. The KOH precipitate contained 56 % ash and the baryta precipitate about 90 %. Since the extracted solids contained about 12 % ash, it is evident that the water extraction removes most (71 %) of the ash of the mould pad. The barium precipitate consisted of about 12 % barium sulphate and about 88 % barium phosphate.

Several peculiar results were noted in making the colour tests on this first water-extract: the development of a deep green colour with the biuret reagents, and a blue colour with phosphotungstic acid-sodium carbonate. On investigation of the reasons for the blue colour test, it was found that Hunter's [1928] colour test for ergothioneine was strongly positive. Attempts to isolate ergothioneine by two different procedures [Tanret, 1909; Williamson and Meldrum, 1932] failed.

The second water-extract (following the removal of the lipins) differed in a few particulars from the first. It gave a negative biuret test, but positive Millon, Hopkins-Cole and xanthoproteic tests. No proteins were present, however, as was shown by the absence of any precipitate with trichloroacetic acid or upon heating.

Extraction with cold 1 % KOH solution removed about 2/5 of the nitrogen originally present in the mould pad (39·7 %, batch 28; 41·2 %, batch 31). The extract gave positive biuret, xanthoproteic, Millon and Hopkins-Cole tests. A heavy white precipitate was formed upon acidification. A considerable amount of carbohydrate was present in the extract as was evidenced by the fact that the acid hydrolysate of the extract strongly reduced Fehling's solution.

Various procedures were used in an attempt to isolate the protein from the extract, including fractional precipitation by alcohol and acetone, saturation with zinc sulphate, precipitation by trichloroacetic and tungstic acids; but none of these methods gave a protein preparation free from carbohydrate. In fact, the highest nitrogen content found in any fraction was only 12·3 %. It may be that the carbohydrate and protein were combined as in the case of glycoproteins. Other investigators have reported similar low nitrogen figures for mould proteins, 12–12·9 % [Thomas and Moran, 1914; Clutterbuck *et al.*, 1932].

The extraction with boiling 4 % KOH removed a quantity of material which was rather low in nitrogen (4·9–5·8 %). The extract was coloured dark brown, and the precipitates obtained from it were also very dark. Part of the alkali used in the extraction was not accounted for on titration with acid, presumably because of the presence of acidic groups in the extracted material.

The nature of the resistant, alkali-insoluble residue has been discussed elsewhere [Norman and Peterson, 1932]. Hydrolysis for 24 hours with 20 % HCl brought 78 % of its nitrogen into solution. Glucosamine hydrochloride readily crystallised out of the acid hydrolysate and accounted for 62 % of the nitrogen in the resistant residue.

Partition of the water-soluble nitrogen.

The ammonia-nitrogen of the water-extract (batch 28) was very low, only 3·9 % of the total. It is quite obvious that the mould, although it may absorb nitrogen in the form of ammonium salts, does not store it in the hyphae for any length of time in that form.

A rough attempt was made to determine the amount of urea present by precipitation with xanthydrol and determination of the nitrogen content of the precipitate. This was found to be 3·9 % of the total nitrogen in the extract.

The amounts of amide-nitrogen found varied within wide limits according to the method used. The lowest figure, 3·5 %, was obtained by hydrolysis with $2N$ H_2SO_4 for 6 hours [Vickery, 1931]. The highest figure, 14·8 %, was found after hydrolysis with 20 % HCl for 24 hours. The difference between these two figures (11·3 %), is formed from unknown compounds during the severe hydrolysis with 20 % HCl. The maximum figure is slightly less, however, than that reported by Vorbrodt [1926] who found 20·9 % amide-nitrogen in the hydrolysate of the water-extract of *A. niger*.

In some batches of mould the first water-extract contained some substance which had the power of catalytically decomposing nitrous acid to nitric oxide with extreme rapidity. Consequently, attempts to determine the α-amino-nitrogen of these extracts by the Van Slyke method were attended with great difficulty. The apparent amino-nitrogen of these extracts varied from 80 to 110 % of the total nitrogen in the extract. However, in the water-extract of a batch that behaved normally the free amino-nitrogen before acid hydrolysis was 37·5 % of the total nitrogen in the extract. (Table I.)

The nitrogen partition of the water-extract from batches 31 and 34 is given in Table II. The basic nitrogen in the acid-hydrolysed extract varied from 14·9 %

Table II. *Nitrogen partition of water-extract.*

	Batch 31 % of total N	Batch 34 % of total N
Ammonia-N	18·7	18·1
Humin-N	6·6	5·4
Phosphotungstic acid precipitate-N	14·9	20·0
Phosphotungstic acid filtrate, amino-N	58·9	58·4
Phosphotungstic acid filtrate, non-amino-N	1·5	0·3
Total recovered	100·6	102·2

of the total (batch 31) to 20·0 % (batch 34). Vorbrodt [1926] found 6·2 % basic nitrogen in the acid hydrolysate of the water-extract of *A. niger*.

Although the water-extract gave no clearly positive test for peptides, it contained a large proportion of peptide-nitrogen. Before hydrolysis, the α-amino-nitrogen was 30·2 % and after hydrolysis 67·7 %, giving a difference of 37·5 % as combined amino-nitrogen.

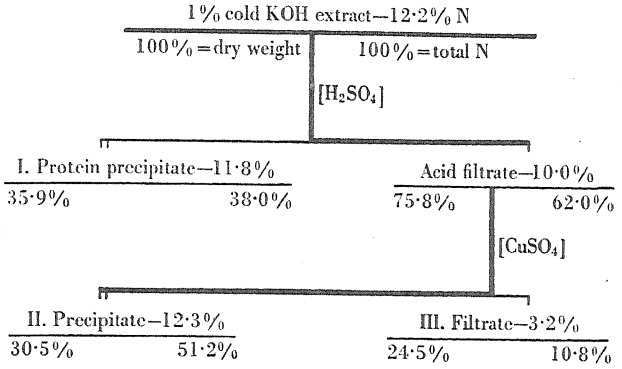
The lipin fraction contained a small amount of nitrogen which was not investigated further.

Fractionation and partition of the alkali-soluble nitrogen.

The cold alkaline extract was found to contain two types of protein—one soluble in acid solution, the other precipitable on acidification. The acid filtrate, however, also contained considerable amounts of carbohydrate, and the acid-soluble protein and carbohydrate had almost identical solubilities, so much so that it was exceedingly difficult to separate the two. For example, tungstic acid, which completely precipitated the protein, was found to precipitate the carbohydrate also.

The procedure finally adopted is shown in the following scheme: precipitation of first protein fraction with acid at p_H 4·8–5·2, precipitation of second protein

fraction with copper sulphate at p_H 6·2–6·5, with most of the carbohydrate remaining in the filtrate.



Fractionation of nitrogen in first alkaline extract (batch 34).

Explanation: Figures above the line and on the right indicate percentage of nitrogen in each fraction (ash-free basis). Figures below the line on the right show the percentage of the total nitrogen which was contained in each fraction. Figures below the line on the left indicate the percentage of the total solids (ash-free) found in each fraction.

The first protein precipitate was a tan-coloured powder; it contained 1·2 % ash, 11·8 % N and 0·4 % P. It was soluble in dilute alkalis, precipitated by acidification with mineral acids (partly dissolved by an excess), acetic, trichloroacetic, phosphotungstic and tungstic acids. It gave all the usual protein colour tests and in addition reduced Fehling's solution after acid hydrolysis.

The copper sulphate precipitate dissolved in excess acid with the formation of a greenish blue colour, and in excess alkali with the formation of a deep purple colour. It contained 12·3 % N and 2·4 % P (ash-free basis), and varying amounts of ash, 12 to 37 %. This preparation was precipitated from acid solution by phosphotungstic and tungstic acids. The acid hydrolysate of this preparation reduced Fehling's solution.

The filtrate from the copper sulphate precipitate no longer gave any protein colour tests but contained 3·2 % N (ash-free basis), of which 36 % was free amino-nitrogen. After acid hydrolysis, it strongly reduced Fehling's solution. The distribution of the nitrogen and of ash-free solids in the three fractions is shown in the scheme.

Table III. *Nitrogen partition of the combined alkaline extracts (batch 31).*

	Extract % of total N	H_2SO_4 ppt. % of total N	$CuSO_4$ ppt. % of total N
Ammonia-N	6·6	4·9	6·7
Humin-N	8·2	7·7	7·9
Phosphotungstic acid precipitate-N	24·4	20·5	31·6
Phosphotungstic acid filtrate, amino-N	51·5	61·5	55·0
Phosphotungstic acid filtrate, non-amino-N	6·1	6·9	0·0
Total recovered	96·8	101·5	101·2

Table III shows the nitrogen partition of the combined alkaline extracts of batch 31, in which 55·5 % of the total N of the mycelium was alkali-soluble. This material differed markedly from the water-extract in that only one-third as much

ammonia was liberated by acid hydrolysis. The alkali-soluble nitrogen also contained a considerably larger amount of basic nitrogen than did the water-extract. The copper sulphate precipitate contained 50 % more basic nitrogen than the acid precipitate, which fact may be due to the presence in the former fraction of most, if not all, of the mould nucleic acid.

This difference between the two fractions was still more marked in the nitrogen partition (Table IV) carried out on the two fractions from the cold 1 % KOH

Table IV. *Nitrogen partition of protein fractions from first alkaline extract (batch 34).*

	H_2SO_4 ppt. % * of total N	$CuSO_4$ ppt. % of total N
Ammonia-N	4·9	8·7
Humin-N	5·8	13·8
Phosphotungstic acid precipitate-N	22·8	38·0
Phosphotungstic acid filtrate, amino-N	60·2	36·5
Phosphotungstic acid filtrate, non-amino-N	3·9	1·8
Total recovered	97·6	98·8

* Average of two determinations.

extract (containing 43·2 % of the total mycelial nitrogen) of batch 34. In the copper sulphate precipitate of this extract 38 % of the nitrogen was basic and only 36·5 % monoamino-nitrogen. This fraction would very likely serve as a convenient source of mould nucleic acid.

Summary.

The solubility and partition of the nitrogenous constituents in the mycelium of *A. fischeri* have been determined. The mould-nitrogen was most easily extracted when fresh, unaltered mould tissue was used. The mycelium contained about 30 % water-soluble non-protein-N, 55 % of alkali-soluble protein-N, and 12 % of alkali-insoluble residue-N.

The alkali-soluble protein consisted chiefly of two fractions, one precipitated by acids, the other acid-soluble but precipitated by copper sulphate.

The acid-insoluble protein contained 1·2 % ash, 11·8 % N and 0·4 % P. Of the N in this protein, 22·8 % was basic N and 60·2 % was monoamino-N.

The acid-soluble protein contained a variable, but generally high, ash (12–37 %), 12·3 % N (ash-free basis), and 2·4 % P (ash-free basis). This protein contained 38·0 % of basic N and only 36·5 % of monoamino-N.

The resistant residue contained 2·3 % N. Of the nitrogen in this residue, 62 % has been isolated as glucosamine-N.

References.

Abderhalden and Rona (1905). *Z. physiol. Chem.* **46**, 179.
Clutterbuck, Lovell and Raistrick (1932). *Biochem. J.* **26**, 1907.
Hunter (1928). *Biochem. J.* **22**, 4.
Norman and Peterson (1932). *Biochem. J.* **26**, 1946.
Peterson, Pruess, Gorcica and Greene (1933). *Ind. Eng. Chem.* **25**, 213.
Pruess, Peterson and Fred (1932). *J. Biol. Chem.* **97**, 483.

Skinner, Peterson and Steenbock (1933). *Biochem. Z.* **267**, 169.
Takata (1929, 1). *J. Soc. Chem. Ind.* Japan, **32**, 155 B.
—— (1929, 2). *J. Soc. Chem. Ind.* Japan, **32**, 243 B.
Tanret (1909). *J. Pharm. Chim.* **30**, 145.
Thomas (1928). *Amer. J. Bot.* **15**, 537.
—— (1930). *Amer. J. Bot.* **17**, 779.
—— and Moran (1914). *Compt. Rend. Acad. Sci.* **159**, 125.
Vickery (1931). *J. Biol. Chem.* **90**, 179.
Vorbrodt (1919). *Bull. Intern. Acad. Polonaise, Classe Sci. Math. Nat.* B., 71.
—— (1921). *Bull. Intern. Acad. Polonaise, Classe Sci. Math. Nat.* B., 223.
—— (1926). *Bull. Intern. Acad. Polonaise, Classe Sci. Math. Nat.* B., 517.
Williamson and Meldrum (1932). *Biochem. J.* **26**, 815.

LXXIV. INTERACTION OF VITAMIN D AND DIETARY FACTORS IN THE HEALING OF RICKETS IN RATS.

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Rickets produced in rats by a high calcium-low phosphorus diet may be healed either by the administration of vitamin D or by the addition of a source of phosphorus to the diet. The estimation of the influence of small additions of sources of phosphorus or of other related changes in composition of the diet has, however, received much less attention than the relation between the degree of healing and the dose of vitamin D. It is of considerable practical importance to find some means of expressing the antirachitic effect of such dietary factors, both alone and in conjunction with vitamin D, more especially in those cases where a measure is to be made of the vitamin D content of foodstuffs containing phosphorus compounds. From the theoretical point of view, the discovery of such a relation would throw some light on the synergism of vitamin D and phosphorus compounds.

The exact form of the effect of added phosphorus compounds on the healing produced by vitamin D may be complicated. However, two simple alternative approximations suggest themselves. The antirachitic activity might be additive, *i.e.* equivalent to that of an additional dose of vitamin D, or it might be multiplicative, *i.e.* multiplying the effect of doses of vitamin D actually administered. Key and Morgan [1932] found that by changing the ratio of calcium to phosphorus from 4 : 1 to 2 : 1 in a certain rachitogenic diet, a healing effect was produced equal to that of a dose of 0·7 International Unit (I.U.) of vitamin D with the original diet. Again, Lecoq and Villette [1933, 2] found the curative dose of vitamin D for rachitic rats on the Randoin-Lecoq diet to be 20 I.U. incorporated in 100 g. of diet. The curative doses of various phosphates had previously been found [Lecoq and Villuis, 1932; Lecoq and Villette, 1933, 1], and they now proceeded to assign to these compounds potencies in antirachitic units. This form of expressing the effect of changes in mineral composition of the diet is convenient and is justifiable when vitamin D is not administered simultaneously; but it is unjustifiable to assume, without further experiment, any particular relation of the combined effect to the effects of the two influences separately. A certain ambiguity may arise in this connection, and it is important to make quite clear the distinction between the sum of the effects, measured separately, of two influences and the effect of their sum when applied simultaneously. The expression of both dietary changes and doses of vitamin D in antirachitic units would seem to imply that these two influences should be summed in order to calculate their combined effect. Both groups of workers do, in fact, conclude that the effect of simultaneous administration of phosphate and of vitamin D will be the sum of their separate effects.

When partial healing is produced by administration of vitamin D to rats on a rachitogenic diet it is commonly recognised that although the general form of

the curve relating dose to healing effect must be S-shaped, the central portion is approximately logarithmic. Thus for a considerable range of dose the degree of healing is proportional to the logarithm of the dose. This relation has been found for radiographic measurements of healing by Everse and Van Niekerk [1931] and by Bourdillon *et al.* [1931], for the line test by R. S. Morgan [1932], for growth response by Coward *et al.* [1932], and also for measurements by the prophylactic method using A/R ratios [Hume *et al.*, 1932] or a radiographic scale [Bourdillon and Bruce, 1932]. In the case of the radiographic scale used by Bourdillon *et al.* [1931], this relation is expressed by the equation

$$\log_{10} D_2 - \log_{10} D_1 = \frac{(n_2 - n_1)\log_{10} 2}{2},$$

where n_1 and n_2 are the scale numbers of litter-mates which have received doses D_1 and D_2. With a homogeneous stock of rats kept under uniform conditions, the effect of animal variation is reduced to a minimum, and the mean degree of healing, n, in a group of rats will be fairly closely proportional to the logarithm of the dose, log D. The graphical relation between n and log D in an ideal case will be a straight line, *e.g.* (*a*) in Fig. 1.

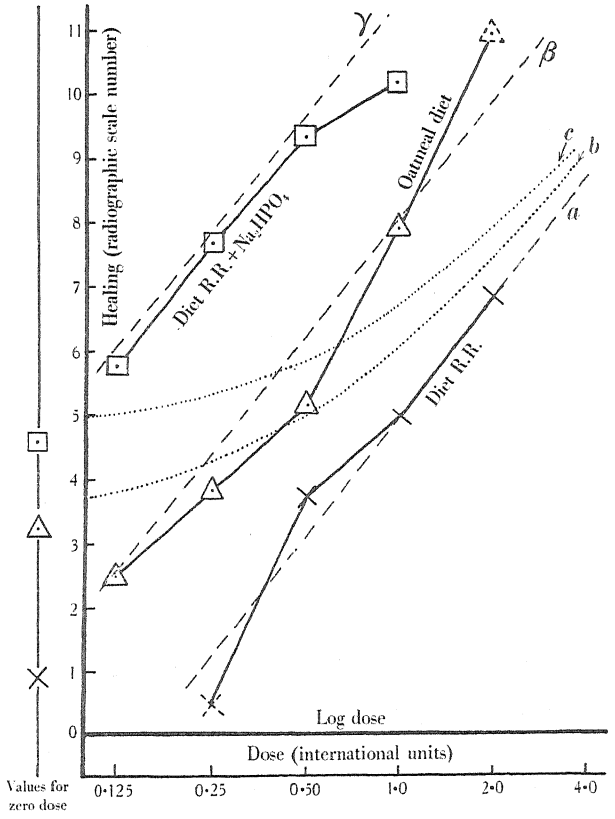

Fig. 1. Comparison of experimental and theoretical relations between log dose and healing.

——— Lines joining experimental points.
— — — Ideal line for diet R.R. (*a*), and lines calculated from this on multiplicative basis (β and γ).
..... Curves calculated on additive basis (*b* and *c*).

If, now, an alteration in the diet is equivalent to the addition of a dose of vitamin D, say d, the relation becomes

$$n \propto \log (D+d),$$

and the relation between the logarithm of the actual dose given and the scale number is no longer linear, *cf.* curves (*b*) and (*c*) in Fig. 1, which deviate considerably from the original line at low values of D, but approach it at high values.

If, however, an alteration in the diet has the effect of multiplying the dose by a factor ϕ, the relation becomes

$$n \propto \log \phi D = \log \phi + \log D.$$

The relation is still linear, but the line is shifted a distance along the n axis corresponding to $\log \phi$, and remains parallel with the line for the original diet, as is shown in Fig. 1, curves β and γ. At higher doses there is a clear and increasing difference between the values given by additive and multiplicative constants, corresponding to the increasing difference between the sum of these two constants and their product. The curves b and c in Fig. 1 show that a constant addition to the actual dose given has a varying effect with different doses, whilst the lines β and γ in Fig. 1 show that multiplication of the dose by a constant factor produces a constant addition to the effect, increasing the healing by the same number of degrees on the scale with any dose.

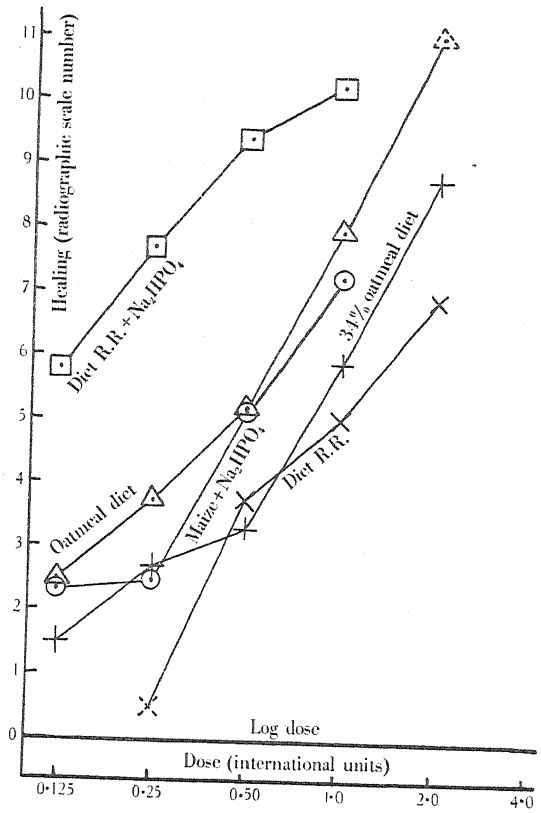

Fig. 2. Relation between log dose and healing. Experimental data for five diets.

In the course of the investigation described in the next paper [Bruce and Callow, 1934] we had occasion to compare quantitatively the calcifying powers of diets containing either different cereals or different forms of phosphorus, and we had to settle the question of how these differences were to be expressed. In Fig. 2 is shown the result of plotting degree of healing against logarithm of the dose with five diets for which suitable data are available. The picture corresponds reasonably well with a series of parallel lines, and there is no resemblance whatever to a series of convergent curves such as would be required by additive influences. Confirmation is afforded by Fig. 1, in which data obtained for the two least rachitogenic diets (oatmeal and R.R. + Na_2HPO_4) are plotted and compared with curves calculated on the basis of additive constants (b and c) or multiplicative factors (β and γ) applied to the standard rachitogenic diet (R.R.). The evidence is decisively in favour of multiplicative factors for both diets.

Experimental.

Data.

Full details of the technique and of the diets used are given in the next paper [Bruce and Callow, 1934]. The data from which the graphs in Figs. 1 and 2 have been constructed are given in Table I.

Table I.

Description of diet	Dose (International Units)	Healing Mean radiographic scale no.	No. of rats
R.R. (routine rachitogenic diet: 20% maize, 48% white flour, 32% basal mixture)	Nil	0·9	4
	(0·25	0·5	2)
	0·5	3·76	15
	1·0	5·02	45
	2·0	6·9	5
34% oatmeal (a mixture of equal parts of R.R. (above) and oatmeal diet (*cf.* below))	Nil	0·33	3
	0·125	1·5	3
	0·25	2·7	3
	0·5	3·3	6
	1·0	5·9	5
	2·0	8·75	4
Maize + Na_2HPO_4 (68% maize, 32% basal mixture, 2·46 g. Na_2HPO_4 (anhydr.) per kg.)	Nil	0·5	4
	0·125	2·33	3
	0·25	2·5	7
	0·5	5·14	18
	1·0	7·22	11
Oatmeal (68% oatmeal, 32% basal mixture)	Nil	3·23	58
	0·125	2·5	4
	0·25	3·85	17
	0·5	5·19	50
	1·0	7·95	17
	(2·0	11·0	2)
R.R. + Na_2HPO_4 (R.R. (above) + 8 g. Na_2HPO_4 (anhydr.) per kg.)	Nil	4·57	31
	0·125	5·79	19
	0·25	7·7	41
	0·5	9·38	13
	1·0	10·25	4

Calculation of additive constants.

A straight line is drawn through the points for diet R.R. in Fig. 1. The doses, as given by this line, corresponding to the scale numbers given by rats on oatmeal diet and on R.R. + Na_2HPO_4 diet with no administered dose, determine the values of d for these diets. The values are $d = 0{\cdot}51$ unit for the oatmeal diet, and

$d=0{\cdot}84$ unit for the R.R.$+Na_2HPO_4$ diet. The theoretical curves (*b*) and (*c*) are then plotted, substituting, at values of the logarithm of the actual dose, values for the scale number corresponding to an assumed dose, $(D+d)$.

Calculation of multiplicative constants.

The values of the multiplicative factor are derived from a series of comparisons of litter mates on diet R.R. and the other two diets, which is described in detail in the paper following this. The relation for the radiographic scale, *viz.*

$$\log_{10} D_2 - \log_{10} D_1 = \frac{(n_2 - n_1)\log_{10} 2}{2}$$

becomes

$$D_2/D_1 = \text{antilog}\,[(n_2 - n_1) \times 0{\cdot}1505].$$

In the case of two diets A and B, the value of D_2/D_1 which is found is an "apparent dose ratio," a_A/a_B. If the ratio of the doses actually administered is d_A/d_B, then the apparent dose ratio is given by

$$\frac{a_A}{a_B} = {}_A\phi_B \times \frac{d_A}{d_B},$$

where ${}_A\phi_B$ is the multiplicative factor for diet A with respect to diet B, and

$${}_A\phi_B = \frac{a_A}{a_B} \times \frac{d_B}{d_A}.$$

Referred to diet R.R., the value for ϕ for the oatmeal diet, determined from the mean of a number of direct comparisons, is 3·2, whilst for the R.R.$+Na_2HPO_4$ diet, $\phi = 8{\cdot}35$.

Summary and Conclusions.

When vitamin D is administered to rachitic rats on a diet containing excess of calcium, the influence of increasing or altering the source of phosphorus may be expressed as a factor multiplying the dose of vitamin D. Expression of the dietary influence as the addition of a certain number of antirachitic units to the actual dose of vitamin D gives figures which are not concordant with experimental results. The combined antirachitic effect of changes in the mineral composition of a diet and of simultaneous administration of vitamin D is thus greater than the effect of the sum of the two acting separately. It is actually more nearly proportional to the product of two factors representing these antirachitic influences.

It is a pleasure to record our thanks to Dr R. B. Bourdillon for his helpful criticism in the course of preparing this work for publication.

REFERENCES.

Bourdillon and Bruce (1932). *Biochem. J.* **26**, 506.
—— Bruce, Fischmann and Webster (1931). *Med. Res. Coun. Sp. Ser.* No. 158. (H.M. Stationery Office, London.)
Bruce and Callow (1934). *Biochem. J.* **28**, 517.
Coward, Key and Morgan (1932). *Biochem. J.* **26**, 585.
Everse and Van Niekerk (1931). *Ned. Tijdschr. Geneesk.* **75**, 1101.
Hume, Pickersgill and Gaffikin (1932). *Biochem. J.* **26**, 488.
Key and Morgan (1932). *Biochem. J.* **26**, 196.
Lecoq and Villette (1933, 1). *Compt. Rend. Soc. Biol.* **112**, 1051.
—— —— (1933, 2). *Ann. Falsif. Fraudes*, **26**, 409.
—— and Villuis (1932). *Compt. Rend. Soc. Biol.* **109**, 539.
Morgan, R. S. (1932). *Biochem. J.* **26**, 1144.

LXXV. CEREALS AND RICKETS. THE RÔLE OF INOSITOLHEXAPHOSPHORIC ACID.

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As the result of a long series of investigations, E. Mellanby [1921; 1922; 1924; 1925; 1926; 1930; 1931; *cf.* Green and Mellanby, 1928] and M. Mellanby [1929] have demonstrated that certain cereals contain an anticalcifying factor having the effect of producing or intensifying rickets in dogs or rats and of retarding calcification of the teeth in dogs. Oatmeal, for instance, was found to have a marked anticalcifying effect in comparison with white flour or rice when the diet contained a large proportion of cereal. M. Mellanby [1929] summarised the evidence as to the nature of this factor as follows: "It has been impossible to obtain evidence to show that the anticalcifying action of cereals on the teeth is due to any known constituent. Neither the fat, carbohydrate, calcium and phosphorus (absolute and relative amounts) nor the acid-base ratio of the mineral constituent explains this action of cereals. There is some evidence that it is associated with protein and liberated from it by proteolytic digestion."

Certain points with regard to these investigations should be noted. The great majority of the experiments were carried out with dogs, which, unlike rats, have a low natural ability to make use of dietary calcium and phosphorus in the absence of vitamin D. It is possible, however, to assume that, with a low level of vitamin D, the Ca/P ratio will have a similar effect in controlling absorption of these elements in dogs and rats. Further, since the effect of adding calcium carbonate was to improve calcification, though only slightly, it seems justifiable to regard the diets as being deficient in calcium. The Ca/P ratio of the cereal has however been over-emphasised in relation to that of the diet as a whole. The cereals used all have calcium contents which are small in comparison with those of the other constituents of the diet, but the phosphorus contents are considerable. Using the figures given by E. and M. Mellanby, it will be found that a diet containing oatmeal (P, 0·392 %) will have a lower Ca/P ratio than a diet containing rice (P, 0·096 %), although the cereals alone have Ca/P ratios of 1 : 5·7 and 1 : 10·7, respectively.

Steenbock *et al.* [1930] compared the calcifying powers of diets containing different cereals with supplements of calcium carbonate and phosphoric acid, using the percentages of ash in the femurs of rats as the basis of comparison. With 1, 2 or 3 % of calcium carbonate in the diet, the calcifying power was in the order: whole wheat > rolled oats > yellow maize, which was the same order as that of the phosphorus contents. Without calcium carbonate, whole wheat showed slightly less calcifying power than the other two cereals. When the phosphorus content was equalised by addition of phosphoric acid, the order of calcifying power of the diets was practically reversed, although the differences were smaller. They say, "Equalisation of the phosphorus content of the cereal rations did not make them equally effective in bone formation, but it did make

the corn (maize) more effective with its $CaCO_3$ supplement. It must however be considered that inorganic phosphorus added as phosphoric acid may not be equivalent in physiological properties to the organic phosphorus compounds in the cereals. Our experiments certainly demonstrate the fact that inequality of phosphorus intake is not the sole reason for the difference in calcifying action of the various cereals."

Fine [1930] also confirmed the existence of differences in the calcifying properties of cereals, but was inclined to ascribe such differences to variations in vitamin D content rather than to variations in content of an anticalcifying factor.

Quite recently György *et al.* [1933] concluded that the existence of a rachitogenic factor in cereals, although lacking material proof, was supported strongly by their results, although they differed from Mellanby in finding that maize meal was most highly rachitogenic. They also confirmed the reduction of the rachitogenic effect by treatment with acid, but showed that the high proportion of sodium chloride resulting from subsequent neutralisation was alone capable of exerting a slight antirachitic effect.

It seemed to us that the influence of absolute and relative amounts of calcium and phosphorus on calcification was worthy of further study. Although there were no grounds for supposing that the elusive anticalcifying factor was entirely a matter of calcium and phosphorus content, a quantitative analysis of the cereal effect from that point of view was a necessary preliminary to further investigation. The suggestion of Steenbock *et al.* that the phosphorus compounds of cereals differed in their biological availability, might, moreover, contain an important clue to the solution of the problem, and attention should be paid to the nature of the phosphorus compounds.

The fact that in the work of Steenbock *et al.* the cereal with the highest phosphorus content had the lowest calcifying power when the phosphorus contents of the diets had been equalised strongly suggests that cereals contain a form of phosphorus less available than phosphoric acid. Independent evidence does, in fact, exist that this is the case.

The mineral components of a diet are too frequently expressed as a total percentage only, with little or no reference to the actual form in which those elements are present. Most cereals are relatively rich in phosphorus, but from 50–80 % of this is present in the form of inositolphosphoric acids, "phytin-phosphorus" [for literature, *cf.* Koehler, 1926], and it is a debatable question whether phosphorus in this form is absorbed at all from the digestive tract. The earlier literature is reviewed by Schaumann [1910]. Starkenstein [1910] concluded from his own investigations on organic phosphates in human urine that only a small proportion of orally administered inositolhexaphosphate was absorbed unchanged, the great part being hydrolysed by bacteria in the intestine. Plimmer [1913, 1] demonstrated that inositolhexaphosphoric acid diffused only slowly through parchment membranes, and that the acid was not hydrolysed by intestinal extracts from carnivora or herbivora. He concluded that there was no evidence of direct absorption, but that after hydrolysis by an enzyme in the foodstuff or by bacterial action it would enter the circulation as inositol and phosphoric acid.

As regards the availability of phytin-phosphorus in rickets, the evidence in the literature is conflicting. Eddy *et al.* [1922] demonstrated that supplements of phytin were ineffective in protecting rats from rickets when added to Sherman-Pappenheimer's diet No. 84. More recently there has been a controversy about the antirachitic activity of "Vitaphos," a preparation of calcium magnesium

inositolhexaphosphate. Von Wendt [1930] claimed that "Vitaphos" contained a form of vitamin D which was not toxic in excessive doses. Vonder Mühll [1931] contested this and demonstrated that the curative effect on rickets in rats could be explained as a result of the addition of phosphorus compounds. A more detailed investigation was recorded by Bleyer and Fischler [1931, 1, 2], who claimed that "Vitaphos" restored the composition of rats' bones to normal when added to McCollum's diet No. 3143 in the proportion of from 3 to 7 %. Their experiments are however open to several objections.

In the present communication there is described an investigation of the differences in calcifying power between cereals and of the availability of phytin-phosphorus, using the method of measuring the calcifying powers of diets which has been described in the preceding paper.

The series of diets compared were all of the high calcium-low phosphorus type, made up from 32 parts of a basal mixture and 68 parts of cereal. In certain cases a source of phosphorus was added. All contained 2 % of calcium. One diet, the "routine rachitogenic diet," had a mean Ca/P ratio of 10·3/1; the others had a mean Ca/P ratio of 5·5/1. In this way a comparison has been made of the effects due to the addition to rachitogenic diets of phosphorus in various forms,

(*a*) as a component of the cereal itself,
(*b*) as disodium hydrogen phosphate, and
(*c*) as sodium or calcium magnesium inositolhexaphosphates.

Further, the effects on oatmeal of cooking and of treatment with dilute acid have been investigated.

It has not been found possible to apply the same method to diets of the low calcium-high phosphorus type, but we have included here an account of some preliminary qualitative comparisons of such diets.

Experimental.

Analytical methods.

The determinations of calcium and phosphorus in dietary constituents and in diets were made by Neumann's method as described by Aron [1912]. Air-dry samples were taken, as variation in the content of moisture affects the absolute figures only slightly, without altering the relative proportions of calcium and phosphorus found.

Phytin-phosphorus (as inositolhexaphosphoric acid) was determined by the method of Heubner and Stadler [1914], with certain of the modifications introduced by Andrews and Bailey [1932].

Diets and materials. The "basal" portion of the diets was composed of:

Wheat gluten	20
Meat powder	6
Calcium carbonate	5
Sodium chloride	1
	32

The components were drawn from the same stocks throughout the investigation. In the "routine rachitogenic diet" (R.R.), and in some of the other experimental diets, the "cereal" portion consisted of a mixture of yellow maize meal (20 parts) and white flour (48 parts); in other diets it consisted of oatmeal or of maize.

The following values were found for certain of the constituents.

Meat powder, Ca, 0·045%; P, 0·75%. Wheat gluten, P, 0·148%. Maize meal, Ca, 0·04%; P, 0·37% (P soluble in HCl, 0·35%; phytin-P, 0·34%; phytin, as $C_6H_{18}O_{24}P_6$, 1·2%.) White flour,

Ca, 0·018%; P, 0·078% (P soluble in HCl, 0·026%; phytin-P, 0·014%; phytin, as $C_6H_{18}O_{24}P_6$, 0·05%). The oatmeal was "Ingles Midlothian Scotch, fine," and contained Ca, 0·06%; P, 0·406% (P soluble in HCl, 0·35%; phytin-P, 0·32%; phytin, as $C_6H_{18}O_{24}P_3$, 1·1%). Disodium hydrogen phosphate was used as the anhydrous salt, dried at 120° and ground to pass 80 mesh. The calcium magnesium inositolhexaphosphate used was a commercial brand of "phytin." Found: Loss at 100°/15 mm., 13·2%, and, referred to anhydrous material, Ca, 11·8%; P, 22·0% (phytin-P, 22·0%; phytin, as $C_6H_{18}O_{24}P_6$, 78·1%). The sodium salt was prepared from this by the method of Posternak [1921], recrystallised from water, dried partially *in vacuo* and ground to pass 80 mesh. (Found: P, 19·2%; phytin-P, 18·5%; inorganic P, 0·7%.) The mixing of small amounts of these addenda with the diets was carried out in batches of 2–4 kg. in a large ball mill for a period of 2·5–3 hours.

We have met with some difficulty in reproducing the exact composition of a diet as regards the total calcium and phosphorus content when the diets have been mixed in bulk. For this reason, where it was necessary to make up more than one batch of any given diet, each batch has been analysed separately. Thus the figures given represent the actual diets which were employed in feeding.

Biological methods.

The practical details of the rat tests were essentially as described by Bourdillon *et al.* [1931]. Rats weaned at approximately 4 weeks of age (50 g. weight) were put on the R.R. diet for 2 weeks, and then the knee-joints were radiographed under anaesthesia to confirm the satisfactory development of rickets. The rats in each litter were then sorted into pairs comparable in weight and sex, as far as was possible, and transferred to the diets to be tested, the two members of a pair being put on different diets. Daily doses of a standardised solution of irradiated ergosterol in olive oil were given in most cases. In general it was arranged that the two members of a pair should have either equal doses or doses which, taking into consideration the differences between the two diets, would produce comparable degrees of healing. 12 doses were given in 14 days. At the end of 14 days of this regime, the rats were killed by coal-gas and the knee-joints again radiographed. The degree of healing in each case was then estimated by comparison with the standard scale.

The general theory of the method used, and its relationship to the method of estimation of vitamin D by radiography has been discussed in the preceding paper [Bruce and Callow, 1934]. For a pair of rats on different diets, A and B, showing healing scale numbers n_2 and n_1, the apparent dose ratio is given by:

$$\frac{a_A}{a_B} = \text{antilog}\,[(n_2 - n_1) \times 0{\cdot}1505].$$

If ${}_A\phi_B$ is the multiplicative factor for diet A with respect to diet B, *i.e.* the influence of diet A in multiplying the apparent magnitude of a dose of vitamin D, the effect of which has been determined for diet B, and if the ratio of the doses actually administered is d_A/d_B, then

$$\frac{a_A}{a_B} = {}_A\phi_B \times \frac{d_A}{d_B},$$

and

$${}_A\phi_B = \frac{a_A}{a_B} \times \frac{d_B}{d_A}.$$

Comparison of high calcium-low phosphorus diets.

In the following section the protocols are given in full in one case, in order to illustrate the method of calculation, but in the other cases only in summary form.

(i) *Comparison of maize + white flour mixture with oatmeal.* Diet R.R. (Ca, 1·97%; P, 0·227%; Ca/P = 8·7) was compared with diet No. 10 (Ca, 2·0%; P, 0·406%; Ca/P = 4·9), in which the cereal portion consisted entirely of oatmeal.

(*a*) In the first experiment, with two litters divided into 8 pairs, the doses of vitamin D in International Units, and the degrees of healing obtained were as given in Table I.

Table I.

	Diet No. 10		Diet R.R.		
Litter No.	Dose (International Units)	Healing. (Scale Units)	Dose. (International Units)	Healing. (Scale Units)	Difference in healing. (No. 10 – R.R.) Δ
384	Nil	2	Nil	1	1
	0·5	4·5	0·5	1·5	3
	1	10	1	5	5
	2	11	2	4	7
1776	Nil	1	Nil	0	1
	0·5	9	0·5	4·5	4·5
	1	10·5	1	7	3·5
	2	11	2	6	5

The mean difference in degrees of healing between rats receiving equal doses of vitamin D on diets No. 10 and R.R. was 3·75, and, from this:

Apparent dose ratio $a_{10}/a_{\text{R.R.}}$ = antilog ($\Delta \times 0{\cdot}1505$) = 3·7.

Since the actual dose ratio, $d_{10}/d_{\text{R.R.}}$, is unity, this apparent dose ratio is equal to ${}_{10}\phi_{\text{R.R.}}$, the ratio of the antirachitic potencies of diets No. 10 and R.R.

(*b*) In the second experiment (Table II) two litters were divided into 5 pairs in which the dose ratio $d_{10}/d_{\text{R.R.}}$ was 1 : 4.

Table II.

	Diet No. 10		Diet R.R.		
Litter No.	Dose. (International Units)	Healing. (Scale Units)	Dose. (International Units)	Healing. (Scale Units)	Difference in healing. (No. 10 – R.R.) Δ
405	0·125	2	0·5	3	– 1
	0·25	5	1	6	– 1
	0·5	5·5	2	6·5	– 1
1822	0·125	1	0·5	4·5	– 3·5
	0·25	5	1	6	– 1

Mean value of $\Delta = -1{\cdot}5$.

$$ {}_{10}\phi_{\text{R.R.}} = \frac{d_{\text{R.R.}}}{d_{10}} \times \frac{a_{10}}{a_{\text{R.R.}}} = \frac{4}{1} \times \text{antilog}\,(-1{\cdot}5 \times 0{\cdot}1505) = 2{\cdot}4. $$

The mean value of ${}_{10}\phi_{\text{R.R.}}$ is 3·2.

(ii) *Addition of calcium magnesium inositolhexaphosphate to maize + white flour mixture.* (*a*) Diet R.R. (Ca, 1·98%; P, 0·195%; Ca/P = 11·5): 8 rats, dose 1 unit; 2 rats, dose 0·5 unit.

Diet No. 18 was made up with the same cereal and base, except that the calcium carbonate was reduced from 5% to 4·77%, and calcium magnesium inositolhexaphosphate was added in the proportion of 27·6 g. to 2993 g. of diet. (Ca, 1·93%; P, 0·364%; Ca/P = 5·3) : 10 rats, dose 0·5 unit.

8 pairs, $d_A/d_B = 0{\cdot}5$, mean $\Delta = -1{\cdot}75$, ${}_{18}\phi_{\text{R.R.}} = 1{\cdot}09$.

2 pairs, $d_A/d_B = 1$, mean $\Delta = +0{\cdot}75$, ${}_{18}\phi_{\text{R.R.}} = 1{\cdot}30$.

${}_{18}\phi_{\text{R.R.}}$ (mean, 10 pairs) = 1·13.

(*b*) Diet R.R. (Ca, 1·90; P, 0·203%; Ca/P = 9·4): 12 rats, dose 1 unit.

Diet No. 24 (equivalent to No. 18) (Ca, 1·94%; P, 0·348%; Ca/P = 5·6) : 10 rats, dose, 1 unit.

10 pairs, $d_A/d_B = 1$, mean $\Delta = 0{\cdot}81$, ${}_{24}\phi_{\text{R.R.}} = 1{\cdot}32$.

For 20 pairs the mean factor for the phytin diet is 1·23.

(iii) *Addition of disodium hydrogen phosphate to maize + white flour mixture.* (*a*) Diet R.R. (as above): 2 rats, dose 0·5 unit; 2 rats, dose 1 unit; 1 rat, dose 2 units.

Diet No. 15, consisting of diet R.R. with the addition of 8·11 g. of Na_2HPO_4 anhydr. per kg. (Ca, 1·90%; P, 0·392%; Ca/P = 4·9): 2 rats, dose 0·125 unit; 2 rats, dose 0·25 unit; 1 rat, dose 0·5 unit.

5 pairs, $d_A/d_B = 0·25$, mean $\Delta = 0·80$, ${}_{15}\phi_{R.R.} = 5·27$.

(*b*) Diet R.R. (Ca, 1·98%; P, 0·195%; Ca/P = 10·2): 7 rats, dose 1 unit.

Diet No. 19 (as No. 15, above) (Ca, 1·98%; P, 0·368%; Ca/P = 5·4): 7 rats, dose 0·25 unit.

7 pairs, $d_A/d_B = 0·25$, mean $\Delta = 2·36$, ${}_{19}\phi_{R.R.} = 9·06$.

(*c*) Diet R.R. (Ca, 1·74%; P, 0·186%; Ca/P = 9·4): 6 rats, dose 1 unit.

Diet No. 21 (as Nos. 15 and 19, above) (Ca, 1·91%; P, 0·359%; Ca/P = 5·3): 6 rats, dose 0·25 unit.

6 pairs, $d_A/d_B = 0·25$, mean $\Delta = 2·67$, ${}_{21}\phi_{R.R.} = 10·08$.

Mean, for all groups, 18 pairs, ${}_{PO_4}\phi_{R.R.} = 8·35$.

(iv) *Comparison of calcium magnesium inositolhexaphosphate with disodium hydrogen phosphate.* Diet No. 18, containing added calcium magnesium inositolhexaphosphate, *cf.* (ii, *a*), above: 12 rats, dose 0·5 unit.

Diet No. 19, containing added Na_2HPO_4, *cf.* (iii, *b*), above: 12 rats, dose 0·25 unit.

12 pairs, $d_A/d_B = 2$, mean $\Delta = 3·5$, ${}_{18}\phi_{19} = 0·15$.

(v) *Comparison of sodium inositolhexaphosphate with disodium hydrogen phosphate.* Diet No. 48 consisting of diet R.R. with the addition of 14 g. of dry sodium inositolhexaphosphate per kg. (Ca, 2·06%; P, 0·420%; Ca/P = 4·9): 4 rats, dose nil; 6 rats, dose 0·25 unit; 6 rats, dose 0·5 unit.

Diet No. 49, consisting of diet R.R. with the addition of 11 g. of anhydrous Na_2HPO_4 per kg. (Ca, 2·12%; P, 0·418%; Ca/P = 5·1): 4 rats, dose nil; 6 rats, dose 0·125 unit; 6 rats, dose 0·25 unit.

4 pairs, dose nil, mean $\Delta = -4·25$, ${}_{48}\phi_{49} = 0·23$.

12 pairs, $d_A/d_B = 2$, mean $\Delta = -2·29$, ${}_{48}\phi_{49} = 0·23$.

Mean, for 16 pairs, ${}_{18}\phi_{49} = 0·23$.

(vi) *Comparison of oatmeal with maize + white flour mixture containing added disodium hydrogen phosphate.* (*a*) Diet No. 13, consisting of diet R.R. with the addition of 8·66 g. of anhydrous Na_2HPO_4 per kg. (Ca, 2·00%; P, 0·433%; Ca/P = 4·6): 6 rats, dose nil.

Diet No. 10, containing oatmeal, *cf.* (i, *a*), above: 6 rats, dose nil.

6 pairs, dose nil, mean $\Delta = 2·92$, ${}_{13}\phi_{10} = 2·75$.

(*b*) Diet No. 15, *cf.* (iii, *a*): 2 rats, dose nil; 2 rats, dose 0·125 unit; 2 rats, dose 0·25 unit; 2 rats, dose 0·5 unit.

Diet No. 10: 2 rats, dose nil; 2 rats, dose 0·125 unit; 2 rats, dose 0·25 unit; 2 rats, dose 0·5 unit.

8 pairs, $d_A/d_B = 1$, mean $\Delta = 2·5$, ${}_{15}\phi_{10} = 2·38$.

(*c*) Diet No. 21, *cf.* (iii, *c*): 4 rats, dose 0·25 unit; 6 rats, dose 0·5 unit; 4 rats, dose 1 unit.

Diet No. 20 (as No. 10) (Ca, 1·97%; P, 0·377%; Ca/P = 5·2): 4 rats, dose 0·25 unit; 6 rats, dose 0·5 unit; 4 rats, dose 1 unit.

14 pairs, $d_A/d_B = 1$, mean $\Delta = 3·89$, ${}_{21}\phi_{20} = 3·85$.

Mean, for all groups, 28 pairs, ${}_{PO_4}\phi_{OATMEAL} = 3·2$.

(*d*) A repetition of (*c*), but with each diet cooked by stirring into four parts of boiling water and heating for half an hour.

Diet No. 21 *c* (phosphate): 4 rats, dose 0·125 unit; 4 rats, dose 0·25 unit; 4 rats, dose 0·5 unit.

Diet No. 20 *c* (oatmeal): 4 rats, dose 0·25 unit; 4 rats, dose 0·5 unit; 4 rats, dose 1 unit.

12 pairs $d_A/d_B = 0·5$, mean $\Delta = 2·04$, ${}_{21c}\phi_{20c} = 4·06$.

(vii) *Comparison of oatmeal with maize + white flour mixture containing added sodium inositolhexaphosphate.* Diet No. 28, consisting of diet R.R. with the addition of 8·5 g. of dry sodium inositolhexaphosphate per kg. (Ca, 1·90%, P, 0·362%; Ca/P = 5·2): 15 rats, dose 0·5 unit.

Diet No. 20, containing oatmeal, *cf.* (vi, *c*), above: 15 rats, dose 0·5 unit.

15 pairs, $d_A/d_B = 1$, mean $\Delta = -0·50$, ${}_{28}\phi_{20} = 0·84$.

(viii) *Comparison of oatmeal with maize.* Diet No. 37, with maize as sole cereal (Ca, 2·00%; P, 0·353%; Ca/P = 5·7): 6 rats, dose 0·5 unit.

Diet No. 38, with oatmeal as sole cereal (Ca, 2·07%; P, 0·379%; Ca/P = 5·5): 6 rats, dose 0·5 unit.

6 pairs, $d_A/d_B = 1$, mean $\Delta = -2{\cdot}83$, ${}_{37}\phi_{38} = 0{\cdot}38$.

(ix) *Comparison of oatmeal with maize and added disodium hydrogen phosphate.* Diet No. 39, with maize as the sole cereal and 2·46 g. of anhydrous Na_2HPO_4 added per kg. (Ca, 2·16%; P, 0·365%; Ca/P = 5·9): 13 rats, dose 0·5 unit; 7 rats, dose 1 unit.

Diet No. 40, with oatmeal as sole cereal (Ca, 2·11%; P, 0·365%; Ca/P = 5·8): 13 rats, dose 0·5 unit; 7 rats, dose 1 unit.

20 pairs, $d_A/d_B = 1$, mean $\Delta = 0{\cdot}20$, ${}_{39}\phi_{40} = 1{\cdot}07$.

(x) *Comparison of cooked, acid-treated and raw oatmeal.* Diet No. 42 was prepared by boiling 4080 g. of oatmeal for four hours with a mixture of 23 litres of water and 1 litre of concentrated hydrochloric acid. After 1·5 hours the product no longer gave a blue colour with iodine. It was then neutralised with anhydrous sodium carbonate (548 g.), the basal mixture (1920 g.) was stirred in, and cooking continued for a further hour. The moisture content of the product was 66%, and analysis, calculated on the oven-dried diet, gave: Ca, 2·25%; P, 0·364%, Ca/P = 6·2; phytin, as $C_3H_{18}O_{24}P_6$, about 0·007%.

Diet No. 41 was prepared by boiling 4080 g. of oatmeal for 4 hours with 24 litres of water then stirring in the basal mixture and an additional 390 g. of NaCl, and cooking for a further hour. The moisture content of the product was 70%, and analysis, calculated on the oven-dried diet gave: Ca, 2·19%; P, 0·364%; Ca/P = 6·0; phytin, as $C_6H_{18}O_{24}P_6$, 0·64%.

Diet No. 43 was the usual dry mixture, with oatmeal as the sole cereal, but with an additional 56·7 g. of NaCl per kg. (Ca, 1·96%; P, 0·324%; Ca/P = 6·1; phytin, as $C_6H_{18}O_{24}P_6$, 0·66%).

A simultaneous comparison was made on triads of litter-mates, without doses, with 21 rats on diet No. 41, 21 rats on diet No. 42, and 20 rats on diet No. 43.

Diets Nos. 41 and 42, mean $\Delta = 3{\cdot}46$, ${}_{12}\phi_{11} = 3{\cdot}66$.
Diets Nos. 42 and 43, mean $\Delta = 3{\cdot}32$, ${}_{12}\phi_{13} = 3{\cdot}32$.
Diets Nos. 41 and 43, mean $\Delta = 0{\cdot}28$, ${}_{11}\phi_{13} = 0{\cdot}91$.

Comparison of low calcium-high phosphorus diets.

The results obtained from the feeding of low calcium-high phosphorus diets have not been very satisfactory. It was first attempted to produce rickets, suitable for radiographic comparison, either by diets which consisted essentially of those described above, but with the omission of calcium carbonate and with added phosphorus compounds. Only a mild type of rickets was obtained, and the radiographic pictures more nearly resembled those found in prophylactic tests. It was found difficult to secure satisfactory growth on low calcium diets, and a slight tendency towards spontaneous healing was often found from the third week onwards. Three classes of diet were employed, one containing oatmeal and the other two containing maize + white flour mixture with the addition of sodium inositolhexaphosphate or sodium phosphate to bring the total phosphorus content up to the same level. Such slight differences as were observed in the rickets resulting after 14–28 days on these diets indicated that the rachitogenic power of the oatmeal diet was slightly less than that of the other two diets, but differences were so small and variable that no real significance could be attached to the result. Using the type of diet described by Green and Mellanby [1928] we were again only able to produce a mild type of rickets in spite of improved growth, but could not detect a difference between the effect of oatmeal and of maize-white flour mixture.

In another experiment the A/R ratio was used as a measure of calcification. Rats weaned at about 4 weeks old were fed one of three diets containing in common the basal mixture: wheat gluten, 20 parts; meat powder, 6 parts; dried liver, 3 parts; sodium chloride, 1 part; No. 44 contained oatmeal (70 parts),

No. 45 contained white flour (70 parts), and sodium inositolhexaphosphate (1·3 parts), and No. 46 contained white flour (70 parts) and disodium hydrogen phosphate (1·1 parts). The phosphorus contents were practically equal, *viz.* 0·38, 0·36 and 0·38 %, respectively. Rats on each of the diets were killed at intervals of a week, from 4 to 8 weeks after the beginning of the experiment. In Table III are shown the time interval, number of rats killed, and average A/R value.

Table III. *Values of* A/R (*no. of rats in parentheses*).

Time (weeks)	4	5	6	7	8
Diet No. 44 (oatmeal)	0·374 (1)	0·433 (2)	0·374 (2)	0·347 (2)	0·391 (2)
Diet No. 45 (inositol-hexaphosphate)	0·545 (1)	0·384 (2)	0·361 (2)	0·428 (2)	0·495 (2)
Diet No. 46 (phosphate)	0·472 (1)	0·468 (2)	0·458 (2)	0·545 (3)	0·502 (1)

It will be seen that the rats on the phosphate diet, after the fourth week, have a consistently higher value of A/R than the rats on the inositolhexaphosphate diet. The rats on the oatmeal diet have a value of A/R which is generally below the value for the rats on the other diets; this is largely to be accounted for by the fact that the rate of growth was very much higher for these rats than for those on either the phosphate or inositolhexaphosphate diets, and an assessment of the anticalcifying power of the oatmeal diet relative to the other two is not possible. In the two latter cases the rates of growth fell off after the third week. With all three diets the growth was unsatisfactory after the sixth week, and an increase in the A/R ratio occurred.

Discussion.

Results with high calcium diets.

The results of the direct comparisons of high calcium diets (i) with diet R.R., (ii) with diet R.R. containing added phosphate and (iii) with the oatmeal diet are summarised in Table IV, in which the diets in the first column are arranged in order of decreasing antirachitic potency.

Table IV.

Characteristic of Diet A	Ca/P (mean)	${}_A\phi_B$: Diet B; R.R.	${}_A\phi_B$: Diet B; R.R. $+Na_2HPO_4$	${}_A\phi_B$: Diet B; oatmeal
Acid-treated oatmeal	6·2	—	—	3·3
R.R. $+Na_2HPO_4$	5·2	8·35	(1)	3·2
Maize $+Na_2HPO_4$	5·4	—	—	1·1
Oatmeal	5·4	3·2	0·32	(1)
Cooked oatmeal	5·9	—	—	0·91
R.R. + Na inositolhexaphosphate	5·1	—	0·23	0·84
Maize	5·7	—	—	0·38
R.R. + Ca Mg inositolhexaphosphate	5·4	1·2	0·15	—
R.R.	10·3	(1)	0·16	0·32

Considering first the diets which have been directly compared with diet R.R., it is seen that the addition of calcium magnesium inositolhexaphosphate causes quite a small increase in the antirachitic potency of the diet, whilst addition of disodium hydrogen phosphate to give the same content of phosphorus causes a very considerable increase. The oatmeal diet, again with the same total phosphorus content, is in an intermediate position. The comparisons with diet R.R. containing added phosphate confirm these relationships, but the diet with added calcium magnesium inositolhexaphosphate is here slightly less antirachitic than diet R.R., and it is evident that the effect of the addition in these amounts is negligible. Sodium inositolhexaphosphate, on the other hand,

appears to cause a definite increase in antirachitic potency, but it should be noted that the specimen used contained a certain amount of inorganic phosphate.

The oatmeal diet has been compared with five other diets containing the same proportion of cereal and of practically the same composition with respect to calcium and phosphorus. The value of ϕ for acid-treated oatmeal diet is 3·3 when compared with the untreated oatmeal diet. This might equally well be explained either by the assumption that the treatment has rendered the phosphorus more readily available, or by the assumption that treatment with acid has destroyed a rachitogenic factor of some other nature. In support of the first assumption are the facts that inositolphosphates were hydrolysed to phosphates by this treatment and that the availability of the phosphorus was consequently greatly increased. Moreover, the addition of phosphate to diet R.R. yields a diet of antirachitic potency practically equal to that of the acid-treated oatmeal diet with the same phosphorus content.

Confirmation of this view is afforded by comparison of the oatmeal diet with two others which may be regarded as "artificial oatmeal" diets. In the first of these the cereal was maize, with nearly the same proportion of inositolhexaphosphate but with less total phosphorus, and this was brought up to the same level as in oatmeal by the addition of disodium hydrogen phosphate. In the second diet the low phosphorus content of the maize+white flour mixture (estimated at total P, 0·17 %; phytin-P, 0·11 %; other P, 0·06 %) was raised by the addition of sufficient sodium inositolhexaphosphate (containing a little phosphate) to give a mixture (total P, 0·41 %; phytin-P, 0·32 %; other P, 0·09 %) comparable with the oatmeal (total P, 0·41 %; phytin-P, 0·32 %; other P, 0·09 %). The values of ϕ for these two diets, relative to the oatmeal diet, are 1·1 and 0·84 respectively. They are in fact equal to that of the oatmeal diet within the limits of experimental error. It is therefore concluded that, under these conditions, the differences in antirachitic potency between oatmeal and either maize or a maize+white flour mixture (20 : 48) can be practically entirely accounted for by the differences in content of phosphorus in available forms and in the non-available form of inositolhexaphosphate. E. Mellanby [1930], referring to the cereal effect in rickets, said "a greater deficiency of calcium and phosphorus in the body can be brought about by diets which actually contain more of these substances, so that an analysis of mineral intake may be quite misleading as to the efficacy of a diet in adequately supplying these substances to the tissues." The work now described in this paper is in full accordance with this view, in that it has shown that in high calcium diets cereals are apparently more rachitogenic than other material of the same mineral composition, just because their phosphorus content is illusory—the diet is deficient in available phosphorus in spite of being rich in total phosphorus.

Results with low calcium diets.

It is to be regretted that the above experiments are necessarily confined to rats on a high calcium-low phosphorus type of diet, the only conditions under which methods of quantitative comparison of calcifying or anticalcifying action have been worked out. However, the results of the preliminary experiments with rats on low calcium-high phosphorus diets described in this paper, although by no means definitive, give an indication that inositolphosphoric acid has, under such conditions, an anticalcifying action comparable with that of phosphoric acid for the same amount of phosphorus. It is possible to assume that this action is the result of lowering the availability of the calcium by binding it as a calcium salt which is at least as insoluble as calcium phosphate.

Inositolhexaphosphoric acid forms a calcium salt which is sparingly soluble in water, but exact data of its solubility relations are lacking. Under physiological conditions, the experiments of Starkenstein [1914] indicate that the toxicities of orthophosphates and inositolphosphates, which are attributed to their powers of precipitating calcium, are equal in solutions of equal normality ($M/3$ and $M/12$, respectively), when they are administered by mouth or subcutaneously, but that inositolhexaphosphate is three times as toxic as orthophosphate when injected intravenously. Starkenstein suggests that conversion of calcium into a non-ionised form is a factor in this toxic action. In low calcium diets there is thus, in addition to the factor of the low availability of the phosphorus, this fact that inositolhexaphosphoric acid is a precipitant of calcium and tends to reduce the latter to a deficiency level. The exact extent of this tendency is not susceptible of measurement by the methods we have been able to employ, but evidence from other sources shows conclusively that an effect of this kind exists.

The cereal factor in calcification.

It is of particular interest to discuss further the experimental results of M. Mellanby [1929]. The evidence presented with respect to the modifying influence of certain forms of treatment on cereals shows a remarkable parallelism with the known effect of similar treatment on inositolphosphates. The destruction of the anticalcifying factor by boiling oatmeal with 1 % hydrochloric acid, first demonstrated by Green and Mellanby [1928], and its relative stability to sodium hydroxide are comparable with the relative rates of hydrolysis of inositolphosphate by acid and alkali [Plimmer, 1913, 2]. The conclusion that the destruction of the anticalcifying factor during germination and malting may be enzymic is in accordance with the known occurrence of phytase in malt [Adler, 1916]. Further, the possibility of association of the anticalcifying factor with cereal proteins, indicated by digestion experiments on the proteins of oatmeal, recalls the combination of inositolphosphate with protein substances in oatmeal demonstrated by Lindenbaum [1926; *cf.* Koehler, 1926; Mnich, 1931]. It would be of interest to extend experimentally the correlation of anticalcifying properties with the relative proportions of inositolhexaphosphate and phosphate, already demonstrated in this paper in the case of acid treatment of oatmeal.

Vitamin D testing.

The distinction between phytin-phosphorus and other phosphorus compounds in cereals with respect to availability in rachitogenic diets is of significance in connection with certain aspects of vitamin D testing. It has been a common experience in this and other laboratories that unexplained variations in the severity of rickets produced by a rachitogenic diet occur from time to time in spite of a reasonably careful control of the diet [*cf.* Bourdillon *et al.* 1931]. One possible factor in this variation may be variations in the proportions of phytin and other phosphorus in samples of maize of similar total phosphorus content. Moreover, since phytase is present in the grain, the proportion of inorganic phosphate may increase on prolonged storage [*cf.* Minkovska, 1926].

Nervous degeneration.

The word "toxamin" was applied by E. Mellanby to "substances [in cereals] of unknown composition which have harmful effects in the body and can be antagonised by specific vitamins" [E. Mellanby, 1930]. As well as an anticalcifying toxamin, toxamins producing nervous symptoms are believed to be present

in cereals. It seemed worth while investigating whether inositolhexaphosphoric acid, which in these experiments accounted for the anticalcifying toxamin, also played some part in the production of nervous degeneration. This idea was supported, during the course of this work, by the appearance of a paper by Stockman and Johnston [1933], in which they described the extraction from cereals of an acid material which produced symptoms of nervous degeneration in monkeys. It seemed likely, considering their methods, that the material contained inositolhexaphosphoric acid, and this led us to try the effect of sodium inositolhexaphosphate on monkeys. The results obtained have so far been negative and are given in summary form only. Three female *Rhesus* monkeys were used, weighing 2·7–3·1 kg. They were kept under observation for a preliminary period of 3 months and were in good condition when the experiment was started. The monkeys were then dosed by stomach tube. One monkey, which served as control, was given a daily dose of 1 g. of sodium chloride in 100 ml. of water. Doses of 5–10 g. of sodium inositolhexaphosphate in 100 ml. of water daily given to the other two produced only severe diarrhoea and sometimes vomiting. After 2 weeks, the dose was reduced to 1·25 g. and 1·5 g. in 100 ml. daily. At this level no toxic symptoms were observed even after 3 months. For the first 2 weeks of this period, normal diet was continued, after this the intake of calcium and of vitamin A was restricted, and the proportion of cereal (wheat, white maize and rice) was greatly increased.

At no stage in the experiment was there any significant difference in behaviour or growth between the control monkey and the experimental animals. It seems evident that sodium inositolhexaphosphate is not the primary cause of the nervous degeneration and other toxic effects observed by Mellanby and by Stockman and Johnston.

Summary and conclusions.

A method of quantitative comparison of the influence of different cereal diets of the high calcium-low phosphorus type has been applied to the comparison of oatmeal, a maize + white flour mixture and maize.

In the type of diet used, containing a great excess of calcium, the replacement of maize + white flour by oatmeal causes a slight healing of rickets. The concomitant increase in phosphorus content might be assumed to account for this, but when the total phosphorus contents of the two diets are equalised by the addition of disodium hydrogen phosphate to the maize + white flour diet, the healing effect of the latter is considerably greater than that of the oatmeal diet.

This can be accounted for by the fact that a large proportion of the phosphorus present in oatmeal is actually in the form of inositolhexaphosphoric acid, which has been shown to be a poorly available form.

After treatment with 1 % hydrochloric acid the oatmeal diet has an antirachitic potency equal to that of the maize + white flour + phosphate diet, and the hydrolysis of inositolhexaphosphoric acid which takes place in this process affords sufficient explanation of the increased potency.

A series of quantitative comparisons has shown that the differences between oatmeal, maize + white flour, and maize can be completely accounted for by differences in the total phosphorus content and in the proportion of inositolhexaphosphoric acid.

In diets of high calcium content the apparent rachitogenic effect of cereals when compared with other material of the same phosphorus content is due to the fact that the cereal-phosphorus is not in an available form.

These experiments are, it should be emphasised, concerned only with high calcium-low phosphorus diets. Where defect in calcium is the factor limiting calcification, there are grounds for believing that excess of inositolhexaphosphoric acid will interfere with the absorption of calcium in the same way as excess of phosphoric acid. The same quantitative methods used with the high calcium diets cannot be applied here, but preliminary experiments have shown that diets containing excess of phosphate or of phytate are about equal in anti-calcifying power.

The relative proportions of phytin-phosphorus and other phosphorus in cereals is of significance in diets used for vitamin D testing.

Administration of sodium inositolhexaphosphate by mouth to monkeys produced no specific nervous symptoms. Thus it appears that this substance is not related to the toxamins of cereals which produce nervous degeneration.

A preliminary note on the subject of these investigations has been published previously [Bruce and Callow, 1932].

REFERENCES.

Adler (1916). *Biochem. Z.* **75**, 319.
Andrews and Bailey (1932). *Ind. Eng. Chem.* **24**, 80.
Aron (1912). *Abderhalden's Handb. Biochem. Arbeitsmeth.* **1**, 385.
Bourdillon, Bruce, Fischmann and Webster (1931). *Med. Res. Coun. Sp. Rep. Ser.* No. 158. (H.M. Stationery Office, London.)
Bleyer and Fischler (1931, 1). *Deutsch. med. Woch.* **57**, 1741.
—— —— (1931, 2). *Biochem. Z.* **239**, 224.
Bruce and Callow (1932). *Brit. Med. J.* (ii), 172.
—— —— (1934). *Biochem. J.* **28**, 512.
Eddy, Muller and Heft (1922). *J. Biol. Chem.* **50**, Proc. xvi, p. xix.
Fine (1930). *Cereal Chem.* **7**, 456.
Green and Mellanby (1928). *Biochem. J.* **22**, 102.
György, Popoviciu and Sano (1933). *Z. Kinderheilk.* **55**, 442.
Heubner and Stadler (1914). *Biochem. Z.* **64**, 422.
Koehler (1926). *Bull. Intern. Acad. Polonaise*, B, 707.
Lindenbaum (1926). *Bull. Intern. Acad. Polonaise*, B, 1041.
Mellanby, E. (1921). *Med. Res. Coun. Sp. Rep. Ser.* No. 61. (H.M. Stationery Office, London.)
—— (1922). *Brit. Med. J.* (i), 790, 832; (ii), 849.
—— (1924). *Brit. Med. J.* (i), 895.
—— (1925). *Med. Res. Coun. Sp. Rep. Ser.* No. 93. (H.M. Stationery Office, London.)
—— (1926). *J. Physiol.* **61**, xxiv.
—— (1930). *Brit. Med. J.* (i), 677.
—— (1931). *J. Amer. Med. Ass.* **96**, 325.
Mellanby, M. (1929). *Med. Res. Coun. Sp. Rep. Ser.* No. 140. (H.M. Stationery Office, London.)
Minkovska (1926). *Bull. Intern. Acad. Polonaise*, B, 1007.
Mnich (1931). *Bull. Intern. Acad. Polonaise*, B, 123.
Plimmer (1913, 1). *Biochem. J.* **7**, 43.
—— (1913, 2). *Biochem. J.* **7**, 72.
Posternak (1921). *Helv. Chim. Acta*, **4**, 150.
Schaumann (1910). *Arch. Schiffs- u. Tropenhyg.* **14**, Beiheft **8**, 325.
Starkenstein (1910). *Biochem. Z.* **30**, 56.
—— (1914). *Arch. exp. Path. Pharm.* **77**, 45.
Steenbock, Black and Thomas (1930). *J. Biol. Chem.* **85**, 585.
Stockman and Johnston (1933). *J. Hyg.* **33**, 204.
Vonder Mühll (1931). *Schweiz. med. Woch.* **12**, 109.
Von Wendt (1930). *Klin. Woch.* **9**, 166.

LXXVI. THE PHOSPHATASE ACTIVITY OF SPLEEN EXTRACTS.

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THERE have been many references in the literature to the presence of enzymes, capable of synthesising and hydrolysing phosphoric esters, in mammalian tissues. Grosser and Husler [1912] and Forrai [1923] described such an enzyme in the intestine. Robison [1923] described similar enzymes in the bone, kidney, liver, spleen and pancreas and in this and a subsequent communication [Robison and Soames, 1924] suggested a possible function for the enzyme occurring in the bone in relation to bone formation. This suggestion has received support, and much evidence, which has been clearly summarised by Kay [1931], has accumulated in its favour.

Kay [1928, 1; 1931] has concluded that the phosphatases of the bone, kidney, intestinal mucosa and blood-plasma are identical, basing his claims upon the following evidence.

1. The optimum hydrogen ion concentration for the phosphatases of the bone, kidney, intestinal mucosa and the blood-plasma is the same. Thus for each of these tissues, for primary esters of phosphoric acid it is 8·9 [Kay, 1926; 1928, 1], for secondary esters it is 7·2–7·6 [Asakawa, 1929] and for pyrophosphoric acid it is 7·6 [Kay, 1928, 2].

2. All these enzymes hydrolyse β-glycerophosphoric acid more rapidly than the corresponding α-compound [Kay and Lee, 1931].

3. The dissociation constant of the enzyme substrate complex, using sodium β-glycerophosphate as substrate, is the same for all the enzymes under discussion [Michaelis and Menten, 1913, quoted by Kay, 1932].

4. Extracts of these tissues, *i.e.* bone, kidney and intestine, will synthesise phosphoric esters under identical conditions [Kay, 1926; Jenner and Kay, 1931; Roche, 1931].

5. Magnesium ions activate the phosphatases from these tissues under very similar conditions [Jenner and Kay, 1931].

Martland *et al.* [1924] reported the presence of a phosphatase in the red cells. Roche [1931], working in Robison's laboratory, further substantiated this claim by separating the plasma and red cells of whole blood and comparing the phosphatase properties of the two fluids under strictly comparable conditions. He demonstrated differences in the properties. Thus the optimum hydrogen ion concentration for the phosphatase of the red cell was found to be p_H 6·0 as compared with p_H 8·9 for the enzyme of the plasma. Moreover the red cell-enzyme hydrolysed α-glycerophosphoric acid more rapidly than the corresponding β-compound, while the plasma-enzyme hydrolysed the β-compound more rapidly.

Robison and Soames [1924] described experiments upon the p_H-activity of the bone, kidney, spleen, liver and pancreatic extracts. They interpreted their

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results as evidence in favour of the hypothesis that the spleen, liver and pancreas contained a phosphatase different from that of the bone and kidney.

In the present work this suggestion has been more fully investigated, and evidence is adduced to suggest that the liver and spleen contain a phosphatase which possesses properties somewhat different from those of both the bone- and the red cell-enzyme.

Technique.

The tissues used in the following experiments were generally from sheep or bullocks and were obtained from the public slaughterhouse immediately upon the death of the animal and used without delay in the majority of cases. Wherever delay occurred the tissue was stored at 0° until ready for use.

In the case of the dog tissue, I am indebted to Dr A. Hemingway, of the Physiology Department, of the University College, Cardiff, for his generosity in supplying me with tissues from the animals which he was using for other experiments. These animals were killed under anaesthetic, and the tissues were obtained immediately. There was no pre-death treatment which might invalidate the results here reported.

Extracts of tissue were prepared by a method similar to that of Kay [1928, 1]. The tissue was minced and then ground up with sand and chloroform water. In the case of the spleen and liver an active suspension was obtained by grinding up 1 part of tissue with 5 parts of chloroform water. After standing overnight at room temperature, the mixture was filtered through glass-wool and used without further purification.

The general method of the research consisted of buffering the extract with phthalate, borate or glycine buffers to a suitable p_H and then measuring the extent of hydrolysis of α- and β-glycerophosphoric acids at various p_H values and after heating at 37°. In order to control the p_H more efficiently it was found necessary to use three glycerophosphate solutions, the p_H of which had been previously adjusted for use over small acid, neutral or alkaline ranges. Even under these circumstances it was found that the buffer solutions did not accurately maintain solutions containing tissue extract at the nominal hydrogen ion concentration of the buffer used. Consequently it was always found necessary to carry out a p_H determination in conjunction with each phosphorus analysis; p_H was measured by the capillator method.

Inorganic phosphate was estimated by the method of King [1932]. Furthermore in view of the anomalies that glycerophosphate can cause in the determination of inorganic phosphorus [Davies and Cule-Davies, 1932], both the amount of substrate used and the time taken for colour development were carefully controlled as suggested by these authors.

The variations in the phosphatase activity of spleen and liver extracts at various hydrogen ion concentrations.

Table I shows the variation in the extent of hydrolysis of sodium β-glycerophosphate with extracts of spleen and liver at varying hydrogen ion concentrations.

Each tube contained 5·0 ml. of buffer solution, 1·0 ml. of tissue extract and 1·0 ml. of 1·5 % sodium β-glycerophosphate solution. The tubes were heated for 4 hours at 37° and then proteins were removed by the addition of 1·0 ml. of 20 % trichloroacetic acid. Aliquot portions of the protein-free filtrate were taken for analysis.

Table I. *The variation in phosphatase activity of tissue extracts under varying conditions of* p_H.

mg. of P produced

p_H	1·0 ml. of spleen extract	1·0 ml. of liver extract	1·0 ml. of kidney extract	2·0 ml. of plasma	2·0 ml. of lysed red cells
4·2	0·280	0·280	0·115	—	—
4·6	0·346	0·290	0·115	—	0·110
4·8	—	—	0·115	—	—
5·0	0·346	0·260	—	—	0·122
5·2	0·190	0·220	0·110	—	—
5·6	0·126	—	—	—	0·147
6·4	—	0·175	—	0·070	0·206
6·8	—	0·165	0·090	0·130	0·110
7·0	0·046	0·150	—	—	0·125
7·4	—	—	—	—	0·125
7·6	0·046	—	0·258	0·360	—
8·0	—	0·256	—	0·580	—
8·3	0·126	0·320	0·381	0·606	—
8·9	0·250	0·400	0·461	—	—
9·4	0·260	0·500	0·474	—	—
9·6	0·230	0·450	—	—	—

The above figures are corrected for the amount of P initially present in the enzyme preparation. The zero hour controls were spleen, 0·360 mg.; liver, 0·240 mg.; kidney, 0·075 mg.; plasma, 0·070 mg.; red cells, 0·052 mg. P per volume of tissue extract *etc.* indicated in the table.

The plasma and lysed cells were heated at 37° for 20 hours.

From p_H 4·4 to 5·0 extracts of spleen show an increasing activity reaching a maximum about p_H 4·8. At less acid reactions, the activity decreases fairly rapidly reaching an almost negligible value at p_H values near neutrality. From p_H 7·6 an increase of activity occurs and the rate of increase is fairly rapid. A second maximum is reached at or near p_H 9·0.

Liver extracts exhibit the same phenomenon. The activity increases to a maximum about p_H 4·7 and then decreases to a minimum at p_H 7·0, finally reaching a maximum again at p_H 9·0.

For purposes of comparison and control, similar series of observations were carried out with kidney extracts and red cells. These results are incorporated in Table I.

Kidney extract was prepared by the method of Kay [1926] in 5 % suspension. Plasma and cells were separated by centrifuging. The plasma was centrifuged a second time in order to ensure the removal of all traces of blood cells. The cells were washed three or four times with isotonic saline and then lysed with an equal quantity of distilled water. They were allowed to stand at room temperature for 12 hours in order to facilitate hydrolysis of naturally occurring esters of phosphorus.

Kidney extracts showed only one optimum of activity and that at p_H 9·0. Plasma exhibited phosphatase activity only on the alkaline side of neutrality. The red cell-enzyme, however, exhibited its maximum effect on the acid side of neutrality. These results confirm the findings of other workers [Kay, 1926; 1931; Asakawa, 1929; Roche, 1931].

These experiments demonstrate the fact that liver and spleen extracts exhibit two optima of hydrogen ion concentration for phosphatase activity. One occurring at p_H 9·0 and the other at p_H 4·5 to 5·0. This property is characteristic of liver and spleen but not of kidney, plasma or red cells.

The relative activities of spleen extracts at the two optima of hydrogen ion concentration.

The relative activities of spleen extracts at p_H 5·0 and 8·8 are not constant, either from animal to animal in the same species or from species to species (Table II).

Table II. *The relative activities of extracts of the spleen of various animals at* p_H *5·0 and* p_H *8·8.*

$$\text{Ratio} = \frac{\text{Activity at } p_H \text{ 5·0}}{\text{Activity at } p_H \text{ 8·8}}$$

Animal				
Bullock	Calf	Pig	Dog	Lamb
2·30	1·00	9·00	2·00	2·32
2·80	2·70	4·00	2·90	2·09
2·70	4·50	6·00	—	1·48
0·80	4·30	3·20	—	2·26
2·20	1·40	5·50	—	—
1·50	—	1·40	—	—
—	—	5·50	—	—
—	—	3·80	—	—

The most consistent results were found with bullock, lamb and dog spleens. The average value found for 6 bullocks' spleens was 2·1, with maximum and minimum values of 2·8 and 0·8 respectively. The values found for 2 dogs' spleens were 2·0 and 2·9. The average value for the spleens of four lambs was 2·0, with the maximum and minimum values of 2·3 and 1·5.

The variation of this ratio was however most marked in calf and pig spleens. Thus the maximum and minimum values in the spleens of 5 calves were 4·5 and 1·0 and the average was 2·8. Its deviation was even greater with pigs' spleens. In this species the maximum and minimum values were 9·0 and 1·4 respectively, with an average of 6·0.

The effect of magnesium ions upon spleen extracts and red blood cells under varying conditions of hydrogen ion concentration.

Erdtmann [1927; 1928] and Jenner and Kay [1931] claim that magnesium ions exert an activating effect upon kidney- and bone-phosphatase and also upon the red cell-enzyme at p_H 7·6. The effect of magnesium ions upon spleen extracts and also upon the red cell-enzyme has been observed under different conditions of hydrogen ion concentration and compared with the corresponding effect on the bone-enzyme. The results are incorporated in Fig. 1 and demonstrate the effect of 0·006 *M* magnesium chloride upon spleen- and red cell-phosphatase. The broken line curves show the variation in activity of spleen extracts and also lysed red cells in the presence of added magnesium; the continuous line curves the behaviour in the absence of the added substance.

The observations with spleen-phosphatase yielded an unexpected result. The maximum relative effect occurred at p_H values near the neutral point. From p_H 4·5 to 5·5 a slight inhibition occurred and from p_H 6·0 to 9·5 a definite stimulation, although the maximum relative effect occurred between p_H 6·0 and 8·0. Table III illustrates this point.

Similar results were observed with red blood cells. The maximum effect was observed at p_H values near neutrality (see Fig. 1). Plasma, kidney and bone

Table III.

p_H	mg. P produced in the presence of added Mg	mg. P produced in the absence of added Mg
5·0	0·225	0·264
7·4	0·100	0·021
8·9	0·200	0·084

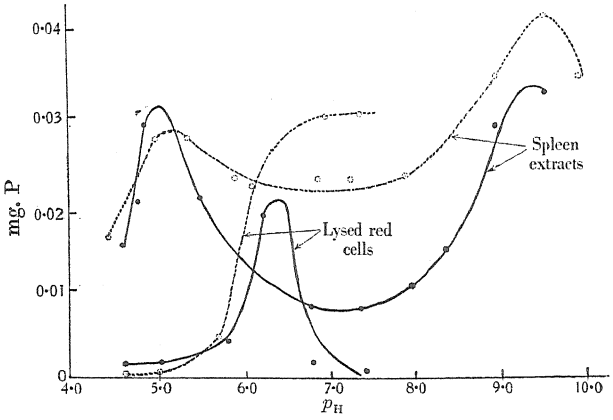

Fig. 1. The hydrolysis of β-glycerophosphate in the presence and absence of added magnesium, by spleen extracts and lysed red cells under varying conditions of hydrogen ion concentration.

●——● Hydrolysis in absence of added Mg; ○ - - - - ○ Hydrolysis in presence of added Mg.

extracts all show marked stimulation by magnesium, particularly with dialysed preparations. The increase in activity varies with the p_H, exhibiting a maximum at the optimum p_H of the enzyme.

Both with blood and spleen, the effect of magnesium seems to be to move the optimum p_H of the enzyme to more physiological values and to facilitate the breakdown of organic phosphates at conditions near neutrality. Inasmuch as this is true, we have a fundamental difference between the enzyme of the blood and spleen on the one hand and that of the bone, kidney *etc.* on the other. The combined enzyme-magnesium complex of the bone-enzyme functions optimally at p_H 8·9, whilst that of the spleen and blood exhibits its maximum effect at or near neutrality.

The hydrolysis of α- and β-glycerophosphates by spleen extracts.

The rates of hydrolysis of α- and β-glycerophosphoric acids in the presence of the bone- and red cell-enzymes are different. The bone-enzyme hydrolyses β-glycerophosphoric acid more rapidly than the α-isomeride [Kay, 1926; Kay and Lee, 1931]. The red cell-enzyme however hydrolyses the α-compound the more rapidly [Roche, 1931].

Fig. 2 shows the variation in the rates of hydrolysis of α- and β-glycerophosphoric acids under various conditions of hydrogen ion concentration, using spleen extracts as the source of enzyme. The β-compound was used as the sodium salt: the α-isomeride was used as the ammonium salt. Solutions were prepared to contain the same concentration of phosphorus.

The α-isomeride was hydrolysed in a similar manner to the β-compound, in that the p_H-activity curve exhibited two optima at the same p_H values as with the β-compound as substrate. From Fig. 2 it will be seen that the ratio of

substrate hydrolysed at p_H 5·0 to that at p_H 9·0 varies with the substrate. Thus with the β-compound the ratio was 0·8 and with the α-compound, using the same extract and at the same time, the ratio was 1·7.

The relative rates of hydrolysis of the two compounds were however quite different. The β-compound was hydrolysed rather faster than the α-compound at alkaline p_H values. Near neutrality, the two substances were hydrolysed at

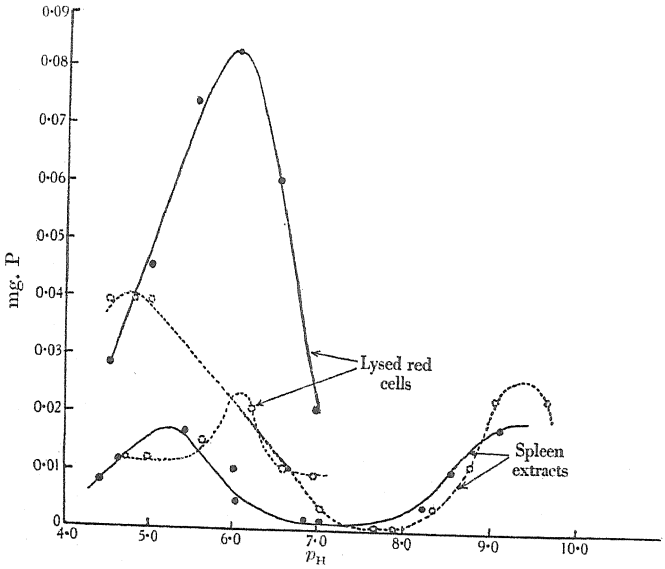

Fig. 2. The hydrolysis of α- and β-glycerophosphates at varying hydrogen ion concentrations, by spleen extracts and lysed red cells.

●——● α-Glycerophosphate; ○- - - -○ β-Glycerophosphate.

nearly the same rate. On the acid side of neutrality however, the β-compound was hydrolysed about three times as fast as the α-compound. In order to control this result the experiment was repeated with red cells. Roche's results were substantiated and a marked difference in the properties of the two enzymes was emphasised (Fig. 2).

The effect of magnesium upon the rate of hydrolysis of α-glycerophosphoric acid was the same as upon that of the β-acid. The relative difference in the rates of hydrolysis was unaltered.

Discussion.

The identity of enzymes cannot be determined exactly as long as one enzyme is mixed with others of the same group. If it is possible to separate the group into its components, then the problem becomes much simpler and the results more definite. The identity of the phosphatases occurring in mammalian tissues cannot be accurately defined, because although results have been quoted in favour of the identity or otherwise of the various phosphatases from animal sources, such results have been based upon the evidence of experiments in which either crude tissue extracts or mixtures of enzymes have been used.

Up to the present, all the reliable evidence suggests the existence of but two distinct and separate phosphatases. (i) The bone-enzyme and (ii) the red blood cell-phosphatase. Roche's claim that the red cell-enzyme is a different mechanism

from that of the bone-phosphatase is a strong one inasmuch as he was able to effect a separation of the enzymes of bone and the red cell and to demonstrate a difference between their properties.

In the present communication experiments have been described which demonstrate the fact that spleen extracts are capable of exhibiting phosphatase activity upon both the acid and alkaline sides of neutrality. The interpretation of such results is difficult because it has not yet been possible to purify the extracts to any great degree, nor has it been possible to separate the extracts into fractions which contain two distinct enzymes. Even so the assumption that spleen extracts contain two distinct and separable enzymes, each capable of hydrolysing phosphoric esters, may be made, in so far as it explains all the facts at our disposal.

Thus such a hypothesis explains the existence of two optima of p_H, the fact that the ratio of the activities of the extracts at the two optima is such a variable quantity (if we are dealing with the same mechanism over the whole range of hydrogen ion concentration studied, then it is reasonable to believe that the ratio should be a constant one); and lastly it explains the effect of magnesium.

The two enzymes may be described for the sake of clarity as the "acid" and "alkaline" phosphatases of the spleen. A comparison of the properties of these two enzymes with those of the bone and blood cells shows some interesting similarities and differences (Table IV).

Table IV. *Comparison of properties of the phosphatases of spleen, bone and red cells.*

	Source of enzyme			
Property	Bone	Red cell	Spleen, acid	Spleen, alkaline
Optimum p_H	8·9	6·0	4·5–5·0	9·0
Effect of magnesium at optimum p_H	Stimulation	Stimulation	Slight inhibition	Stimulation
Effect of magnesium at p_H 7·6	Slight stimulation	Maximum stimulation	Maximum stimulation	Slight stimulation
Relative rates of hydrolysis of α- and β-glycerophosphates	Hydrolyses β-compound 30 % faster than α	Hydrolyses α-compound 300 % faster than β	Hydrolyses β-compound 300 % faster than α	Hydrolyses β-compound about 30 % faster than α

As a result it is possible to identify the alkaline enzyme of the spleen with the bone-enzyme. Thus they both possess the same optimum p_H. They also hydrolyse α- and β-glycerophosphates at the same relative rates, and the effect of magnesium is the same upon both, at both the optimum p_H and at p_H 7·6. The acid enzyme of the spleen however, exhibits markedly different properties from either the alkaline enzyme or the bone-enzyme (Table IV). It resembles the red cell-phosphatase but also differs from this chiefly in two respects; it possesses a different optimum p_H, and it hydrolyses α- and β-glycerophosphates at different rates from the red cell-phosphatase.

The fact that the acid enzyme of the spleen differs from the red cell-phosphatase in just those properties which Kay used for grouping the phosphatases of the bone, kidney *etc.*, suggests the possibility that the two mechanisms may be different. Since, however, little is known of the effect of naturally occurring stimulants and retardants to phosphatase activity it must be remembered that possibly the differences here described may be due to the effect of such substances.

SUMMARY.

1. Extracts of spleen and liver show two optima of hydrogen ion concentration for phosphatase activity. Extracts of bone and intestine and blood-plasma and red cells show only one optimum.

2. The two optima for spleen and liver extracts occur at p_H 4·5–5·0 and at p_H 8·9.

3. There is no relationship between the phosphatase activity at the two optima either from animal to animal or from species to species.

4. Magnesium ions stimulate spleen-phosphatases from p_H 6·0 to 9·5. Below p_H 6·0 a slight inhibition is produced.

5. The maximum relative effect of magnesium ions is found at p_H values near neutrality.

6. Spleen extracts hydrolyse β-glycerophosphoric acid at p_H 5·0 about three times as rapidly as the corresponding α-compound.

7. It is suggested that the spleen contains two separate and distinct phosphatases, one which is identical with the bone-enzyme and the other which possesses properties different from those of the enzyme of either bone or the red cell.

REFERENCES.

Asakawa (1929). *J. Biochem.* **11**, 143.
Davies and Cule-Davies (1932). *Biochem. J.* **26**, 2046.
Erdtmann (1927). *Z. physiol. Chem.* **172**, 182.
—— (1928). *Z. physiol. Chem.* **177**, 211.
Forrai (1923). *Biochem. Z.* **142**, 282.
Grosser and Husler (1912). *Biochem. Z.* **29**, 1.
Jenner and Kay (1931). *J. Biol. Chem.* **93**, 733.
Kay (1926). *Biochem. J.* **20**, 791.
—— (1928, 1). *Biochem. J.* **22**, 855.
—— (1928, 2). *Biochem. J.* **22**, 1446.
—— (1931). *J. Biol. Chem.* **89**, 249.
—— (1932). *Physiol. Rev.* **12**, 384.
—— and Lee (1931). *J. Biol. Chem.* **91**, 135.
King (1932). *Biochem. J.* **26**, 292.
Martland, Hansman and Robison (1924). *Biochem. J.* **18**, 1152.
Robison (1923). *Biochem. J.* **17**, 286.
—— and Soames (1924). *Biochem. J.* **18**, 740.
Roche (1931). *Biochem. J.* **25**, 1724.

LXXVII. THE METABOLISM OF NORMAL AND TUMOUR TISSUE.

XII. THE ACTION OF PHENYLHYDRAZINE ON THE PASTEUR REACTION AND ON TISSUE RESPIRATION.

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THE most pronounced feature of the metabolism of the cancer cell is its property of aerobic glycolysis. With the possible exception of a few spontaneous mouse tumours, where the metabolism of the cancer cells may be masked by that of the connective tissues, all tumours yet examined continued to form lactic acid under aerobic conditions [Warburg, 1930]. The aerobic glycolysis is an expression of a deficiency in the tumour respiration, characterised by a lowering of the R.Q. of the tumour tissue to a level below the carbohydrate value of R.Q. found in normal tissues with similar powers of glycolysis [Dickens and Šimer, 1930, 2; 1931, 2] whether or not the total respiration is diminished in extent. The appearance of aerobic glycolysis shows that in such tissues the mechanism, called by Warburg the Pasteur reaction, which normally correlates respiration and glycolysis, is defective; for normally in a wide variety of aerobic animal and vegetable cells there is a virtually complete suppression in oxygen of the formation of products of the cleavage type of metabolism.

There are two methods by which this problem can be more closely investigated; namely either by the study of methods of artificial production of the property of aerobic glycolysis or by attempts to restore the damaged metabolism to normal. The second of these possibilities is the more difficult to achieve and experiments on this point are in progress.

The most comprehensive study of the conditions under which aerobic glycolysis may be artificially produced is due to Warburg, whose summarised results are as follows [see Warburg, 1929]. I. Inhibition of the respiration; reversibly by means of HCN, irreversibly by temporary asphyxia by keeping the tissue in nitrogen. Spontaneous loss of respiration associated with loss of nuclear material (red blood cell) and accompanying a change of cellular type (embryonic lens). II. Inhibition of the Pasteur reaction, reversibly by ethyl *iso*cyanide, irreversibly by heat and, partially, by exposure of certain embryonic and germinal tissues to salt solutions. To these we may now add certain agents which, like ethyl *iso*cyanide, may be classed under the rather comprehensive title of "heavy metal reagents": 1-amino-2-naphthol-6-sulphonic acid [Krah, 1930], and also reduced glutathione which, according to Bumm and Appel [1932] specifically and reversibly inhibits the Pasteur reaction.

Warburg [1926] pointed out that the poisoning action of ethyl *iso*cyanide on the Pasteur reaction favours the theory of a heavy metal catalyst; the metals

in consideration being iron or, less probably, manganese. Since, on this theory ethyl *iso*cyanide reacts with the catalyst of the Pasteur reaction but not with the respiratory catalyst, whilst the same compound is without action on methaemoglobin but reacts with haemoglobin, the further view that the catalyst of the Pasteur reaction may contain iron in the ferrous state was tentatively suggested. Krah concludes from the action of the reagent mentioned that he has demonstrated the ferrous nature of the catalyst.

In the present paper the action of phenylhydrazine on tissue metabolism is investigated. Phenylhydrazine belongs, according to Lipschitz [1924] to the class of "indirect blood poisons" which are able to oxidise blood pigments only in the presence of oxygen, becoming themselves oxidised in the process. Warburg *et al.* [1931] showed clearly that in oxygen, but not in inert gas (CO), erythrocytes treated with phenylhydrazine become brown in colour, without spectroscopically detectable methaemoglobin formation, whilst on haemolysis there is a heavy deposition of denatured globin, whose production is presumably accompanied by liberation of haematin; for on reduction in an atmosphere of CO the amount of gas taken up by the phenylhydrazine-treated cells was equal to their loss of oxygen capacity. On shaking these cells with air the spectrum of methaemoglobin slowly appears owing to the catalytic action of the haematin on part of the free haemoglobin, and by the reduction of this methaemoglobin sugar can be oxidised in the cells. The treated cells thus show an ability to oxidise glucose which is absent from untreated ones, this property being dependent on the high content of haemoglobin in the starting material. Such an increased sugar oxidation could not therefore be expected to occur in other cells. On the other hand it was of importance to test the reaction upon ordinary body cells of this substance which reacts in the presence of oxygen with compounds such as haemoglobin containing ferrous iron, destroying the complex and causing denaturation of the associated protein. A substance possessing these properties might be expected to influence the Pasteur reaction, since reagents which react within the cell with ferrous iron (ethyl *iso*cyanide, heavy metal reagents of Krah) or denaturation of protein (heat, salt solutions) are known to interrupt the connection between respiration and glycolysis.

In the present paper it is shown that phenylhydrazine, in low concentration, acts as what may be termed an "indirect cell poison," in that in the presence of oxygen, but not in its absence, it is capable of bringing about an aerobic glycolysis which can rise to the anaerobic level. Its action is irreversible, and the substance is without appreciable effect on the anaerobic glycolysis. Whilst with some tissues the respiration is little affected, or even slightly increased, with others it is inhibited to a greater or less extent. The significance of this will be discussed after the description of the experiments.

Experimental.

Methods. The tissue, usually of the rat and in thin razor-slices where necessary, was well rinsed with three changes of Ringer solution, to remove as much of the loosely adhering blood as possible, and was suspended in glucose-bicarbonate-Ringer solution at room temperature and containing sufficient of the $M/20$ solution of phenylhydrazine described below to give the final concentration stated (usually $2{\cdot}5 \times 10^{-3} M$). A control portion of the same tissue was suspended in the untreated Ringer solution. Oxygen containing 5 % CO_2 was allowed to bubble briskly through the suspensions; this pre-treatment usually lasted for 15 minutes. The tissue was then removed, well rinsed first in salt solution,

afterwards for a few minutes in bicarbonate—or phosphate—Ringer while passing the appropriate gas mixture (O_2, or 5 % CO_2 in O_2). It was then transferred to the manometer-vessels containing bicarbonate—or phosphate—Ringer solution with 0·2 % glucose.

Phenylhydrazine. The colourless, recrystallised hydrochloride (B.D.H. "A.R." 73 mg.) was dissolved in 5 ml. water, and made up to 10 ml. with salt-solution and the addition of 0·5 ml. *N* NaOH. This solution (*M*/20) was prepared freshly for each experiment.

Measurement of metabolism. For short experiments, and where the course of the metabolism with time was to be followed, Warburg's [1924] two vessel method was used. Unfortunately with this method not only must a value of R.Q. be assumed in the calculation of aerobic glycolysis, but with increasing value of aerobic glycolysis relative to respiration, the values of the observed pressure changes in the two vessels become more nearly equal, and it follows that the difficulty of obtaining accurate values of respiration with such tissue becomes increasingly great. The technical difficulty of obtaining an accurate measure of respiration in bicarbonate media presents a serious problem with such tissues. To supplement the respiration figures, where the material available was sufficient, the methods of Dickens and Šimer [1931, 1] and Dickens and Greville [1933, 3] have been used. Both need a fairly prolonged experimental time, but the values of respiration, and with the second method of glycolysis, obtained are free from the objection inherent in the use of two different tissue-slices for measurement. The value of aerobic glycolysis in some extreme examples of phenylhydrazine-treated tissue is so great compared with the respiration that no existing method is adequate for really accurate measure of their R.Q. in bicarbonate, the whole extent of the manometer scale being used up by the glycolysis before a sufficiently great respiration had occurred. Finally, measurements of respiration in phosphate-Ringer were made by the method of Dickens and Šimer [1930, 1], but these, while free from such disadvantages, are in a less physiological medium, in which the respiration may not always be the same as in the bicarbonate medium used for the measurement of glycolysis. In the experiments with yeast, where the cells were suspended in acid phosphate, with or without potash as CO_2 absorbent in the vessels, these difficulties did not of course arise. In spite of the technical difficulties mentioned, it is believed that the precautions taken were adequate to ensure reliable measurement in all cases, though in the experiments made by Warburg's method with these highly glycolysing tissues importance should not be attached to minor variations in respiration.

The measurements are collected in Tables I, II and III, and will be briefly surveyed.

Table I. *Embryonic tissues.*

		Control			Treated phenylhydrazine: Pre-treatment						
Exp.	Time (mins.)	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	Conc. *M*/lit.	Time mins.	Exp. time mins.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	Medium
Mouse outer membrane	—	12·9	5·9	—	3 $\times 10^{-3}$	13	—	14·3	20·6	26·0	B
Mouse placenta	—	—	—	—	3 $\times 10^{-3}$	13	—	8·2	5·2	6·5	B
Mouse embryo	—	20·0	14·5	31·0	3 $\times 10^{-3}$	12	30	16·0	22·5	25·0	B
Guinea-pig outer membrane	—	—	—	—	2·5 $\times 10^{-3}$	15	—	12·3	16·0	20·6	B
Rat outer membrane	90	14·1	—	—	3 $\times 10^{-3}$	15	90	9·3	—	—	Phosphate
	60	15·5	6·2	26·0			60	10·2	31·8	28·5	B
Rat outer membrane	40	9·8	3·2	18·7	3 $\times 10^{-3}$	15	40	7·3	17·3	20·0	B
							120	5·5	—	—	Phosphate
Rat embryo (3 mg. dry wt.)	40	11·3	8·4	14·2	3 $\times 10^{-3}$	10	40	14·1	16·2	16·1	B

Table II.

Exp. no.	Time mins.	Control Q_{O_2}	Control $Q_M^{O_2}$	Control $Q_M^{N_2}$	Treated phenylhydrazine: Pre-treatment conc. M/lit.	Pre-treatment Time mins.	Exp. time mins.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	Medium
					Rat testis						
1	60	10·8	2·5	7·3	$2·5 \times 10^{-3}$ (no glucose)	8	60	9·4	6·3	8·3	B
	60	9·6	3·5	11·0	$2·5 \times 10^{-3}$	5	60	14·1	6·9	—	B
						15	60	10·6	8·4	9·2	B
3	30	12·0	4·3	12·0	$2·5 \times 10^{-3}$ (tissue not washed)	15	30	9·4	2·9	11·6	B
		10·4	3·9	—	$2·5 \times 10^{-3}$ (tissue slightly rinsed)	15	30	10·3	6·3	—	B
4	30	12·8	4·8	11·0	$2·5 \times 10^{-3}$ (tissue in two changes Ph.NH.NH_2)	15	30	9·9	11·0	10·1	B
	100	9·9	3·5	—	$2·5 \times 10^{-3}$		100	6·0	7·0	—	B
5	1st 30	13·3	5·0	10·6	,,	15	1st 30	15·5	8·9	8·6	B
	next 60	10·3	3·3	10·6			next 60	12·3	6·2	8·6	
6	—	—	—	—	,,	15	120	6·5	4·7	6·6	B†, R.Q. 0·80
7	30	10·3	5·5	9·0	,,	15	30	15·1	9·0	9·0	B
8	120	10·6	2·3	—	R.Q. 0·98 ,,	15	80	8·2	6·4	—	B
								8·6	7·7	—	B
								9·6	7·1	—	B
							120	8·8	6·2	8·1	B†, R.Q. 0·90
					Rat liver						
	120	11·8	0·2	3·0	R.Q. 0·83 ,,	15	120	8·2	0·5	2·9	B†, R.Q. 0·88

Abbreviations: B = method of Warburg [1924]; B* = method of Dickens and Šimer [1931, 1]; B† = method of Dickens and Greville [1933, 3]; Phosphate = method of Dickens and Šimer [1930, 1].

Table III. *Jensen rat sarcoma.*

Exp. time mins.	Control Q_{O_2}	Control $Q_M^{O_2}$	Control $Q_M^{N_2}$	R.Q.	Treated phenylhydrazine: Pre-treatment $2·5 \times 10^{-3}$ M Ph.$NH.NH_2$ for 15 mins. in all exps.	Exp. time mins.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	R.Q.	Method
30	11·2	24·9	37·5	—		30	13·3	40·5	36·5	—	B
							12·9	36·2	36·5	—	
90	6·8	17·4	30·2	—		90	5·9	28·6	31·8	—	B†
90	—	—	32·0	—		90	5·6	28·0	34·0	0·7	B†
90	8·4	16·7	27·6	—		90	13·0	27·3	—	—	B
							9·7	24·2	27·6	—	B
30	13·3	20·6	35·7	—		30	15·8	32·5	34·8	—	B
150	13·2	18·0	—	0·73		150	9·0	—	—	0·75	B*
							10·3	(25)	—	0·69	B*
150	9·3	—	—	0·70		150	7·6	—	—	0·77	Phosphate

Abbreviations: B = method of Warburg [1924]; B* = method of Dickens and Šimer [1931, 1]; B† = method of Dickens and Greville [1933, 3]; Phosphate = method of Dickens and Šimer [1930, 1].

Effect of phenylhydrazine on the anaerobic glycolysis. The figures given for $Q_M^{N_2}$ in the tables show the effect of phenylhydrazine on the anaerobic glycolysis of a number of tissues. In some cases a slight fall of glycolysis is observed, but the variation is not as a rule much greater than may be found with duplicate pieces of control tissue after similar pre-treatment but without phenylhydrazine. The tables show that on the whole the pre-treatment with phenylhydrazine in the concentration used is without appreciable effect on the glycolysis measured under anaerobic conditions.

Effect of phenylhydrazine on the aerobic glycolysis. In all the tissues used (with the exception of liver, where the glycolysis is small under ordinary conditions) the effect of treatment with phenylhydrazine is to increase the aerobic glycolysis ($Q_M^{O_2}$) very markedly. In most cases $Q_M^{O_2}$ rises to a level very near to the anaerobic value. This is best seen with tissues showing high $Q_M^{N_2}$, such as the embryonic tissues (Table I), and tumour tissue (Table III). As is well known, many of the embryonic tissues show an appreciable glycolysis in Ringer solution which is not observed when the measurements are made in serum or in amniotic fluid: very young embryos may also glycolyse even in these media unless the enveloping membranes are intact [Negelein, 1925]. Even in such cases (Table I) the value of $Q_M^{O_2}$ found in Ringer solution is greatly increased by pre-treatment with phenylhydrazine.

Of tissues with an intermediate degree of glycolysis, testis (Table II) provides a suitable example. Unlike the other tissues studied, for some unknown reason apparently connected with the animals used, testicular tissue of the rat suspended in Ringer solution shows a very variable aerobic glycolysis. Thus Warburg *et al.* [1924] give the following figures (based on R.Q. = 1) $Q_{O_2} -12{\cdot}3$, $Q_M^{O_2} +7{\cdot}2$, $Q_M^{N_2} +8{\cdot}5$; and a similar though less pronounced aerobic glycolysis was observed by Dickens and Greville [1933, 1]. On the other hand, in earlier experiments Dickens and Šimer [1931, 2] found much lower values of $Q_M^{O_2}$, and these are similar to the figures given in the present paper. At the other extreme, Barron [1930] reports that though manometrically he observed a small acid formation ($Q_M^{O_2} +1{\cdot}2$), chemically (in phosphate) he was unable to detect any aerobic lactic acid formation in salt solutions with the specimens of rat testis used by him. Since in manometric experiments we have invariably observed some aerobic acid production with this tissue, it was considered desirable to compare the manometric readings with chemical estimations of lactic acid formed. As the result (Table IV) of the analysis (Friedemann and Kendall's [1929]

Table IV. *Lactic acid estimations.*

	Rat testis			
	mg. dry wt. tissue	Time mins.	Manometrically found mg. acid	Chemical estimation mg. lactic acid
Control tissue I	13·6	120	0·39	0·45
	14·8		0·42	0·38
Control tissue II	22·9	100	0·69	0·62
	21·1		0·64	0·70
Phenylhydrazine tissue I	13·8	120	0·78	0·70
	15·8		0·68	0·66
Phenylhydrazine tissue II	24·3	100	1·23	1·12
	8·65	180	1·00	1·08
	Jensen sarcoma			
Control tissue	12·17	90	1·22	1·07
	11·43		1·15	0·96
Phenylhydrazine tissue	8·05	90	1·32	1·25
	8·72		1·43	1·14
	8·68	90	1·25	1·32

modification of Clausen's method), some 90 % of the manometrically observed acid appeared as lactic acid. Since this is almost within the limits of error with a tissue of such moderate glycolysis, there does not seem any reason to doubt that this is also a true lactic acid fermentation. Thus there appears to be a real

difference between the behaviour of rat testis as studied by different workers for which no explanation appears sufficient at the present time.

These considerations however do not affect the present experiments, where control and phenylhydrazine portions of the same tissue were always compared side by side. As will be seen from Table II, phenylhydrazine had a similar effect on aerobic glycolysis to that shown by the embryonic tissues; the value of $Q_M^{O_2}$ rises after treatment to a higher level, near or equal to $Q_M^{N_2}$.

A further type was provided by tumour tissue. Well-growing non-necrotic specimens of Jensen rat sarcoma (derived from a stock tumour kindly supplied by Miss Scott, Barnato Joel Research Laboratories, Middlesex Hospital, London) were used in all experiments. All methods of measurement of metabolism showed the same result, the already pronounced aerobic glycolysis shown by the Jensen sarcoma is greatly increased in the phenylhydrazine-treated tissue (Table III) and reaches a value which is, within the limits of error, equal to the glycolysis of the same tissue in absence of oxygen.

Nature of the acid formed. The possibility that the acid formed in the manometric observations might not have been lactic acid has been excluded by analysis. For this purpose rat testis and Jensen sarcoma were used and the manometric readings compared with Clausen estimations of the lactic acid formed in the vessels, the results (Table IV) show that some 90 % of the acid formed is lactic acid.

Conditions affecting the action of phenylhydrazine on the metabolism. (*a*) *Washing.* Since phenylhydrazine is known to react with haemoglobin under similar conditions to those in which it reacts with the cell-ferments, it is not surprising that incomplete washing of the tissue reduces the extent of its action. It has been observed that in several experiments where completely unwashed testis, for example, has been used phenylhydrazine ($2{\cdot}5 \times 10^{-3} M$) had no measurable action on the metabolism. If, however, the same tissue were treated with a second portion of the phenylhydrazine Ringer solution, the usual increase of aerobic glycolysis was produced (Table II). This point has not been further studied, well washed tissues being always used in subsequent experiments.

(*b*) *Time and temperature.* At room temperature the reaction with phenylhydrazine is not instantaneous but is usually complete in 15 minutes. Exp. 2, Table II, performed with rat testis illustrates this point.

The reaction also occurs at 37° and is not dependent on the presence of glucose in the medium as the following experiments show.

Jensen rat sarcoma. Temperature 37°. Control, $Q_M^{O_2} + 23{\cdot}5$; the same tissue with $1{\cdot}7 \times 10^{-3} M$ phenylhydrazine in vessel (result, corrected for autoxidation of phenylhydrazine) $Q_M^{O_2} + 37$.

Rat testis. Pre-treatment without addition of glucose to the Ringer-bicarbonate used: 8 minutes at room temperature:

	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$
Control	−10·8	2·5	7·3
With $2{\cdot}5 \times 10^{-3} M$ phenylhydrazine	− 9·9	6·3	8·3
	− 9·0	6·3	—

(*c*) *Oxidative nature of the reaction.* The following experiments show that the action of phenylhydrazine occurs only in the presence of oxygen.

The pre-treatment was carried out at room temperature in the closed vessels attached to their manometers; these were filled with either nitrogen + 5 % CO_2, or oxygen + 5 % CO_2 and were shaken as usual. The phenylhydrazine solution (0·2 ml. $M/20$) was contained in the side-bulb, while the tissue, suspended in

Ringer-bicarbonate-glucose solution (4 ml.) was in the main part. The solutions were first saturated with the appropriate gas mixtures. In the experiments in nitrogen, chromous chloride solution (0·3 ml.) was contained in the central well as oxygen absorbent. The vessels containing solutions and tissue were first shaken for 10 minutes to allow absorption of any residual oxygen in the anaerobic experiments, the phenylhydrazine was tipped in from the bulbs, and the shaking was continued for 15 minutes. The tissue was then quickly removed, rinsed and transferred to the vessels used for measurement of metabolism. It was noted that the colour of small traces of blood present changed to brown in the oxygenated phenylhydrazine portions only.

	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$
Rat testis			
Control: pre-treatment in $O_2+5\%$ CO_2	−10·3	5·5	9·0
Ph.NH.NH_2: pre-treatment in $O_2+5\%$ CO_2	−15·1	9·0	—
Control: pre-treatment in $N_2+5\%$ CO_2	−12·8	5·5	—
Ph.NH.NH_2: pre-treatment in $N_2+5\%$ CO_2	−12·5	5·8	—
Jensen sarcoma			
Control: pre-treatment in $O_2+5\%$ CO_2	—	—	32·4
Ph.NH.NH_2: pre-treatment in $O_2+5\%$ CO_2	− 9·5	31·0	—
	− 7·7	27·8	—
Ph.NH.NH_2: pre-treatment in $N_2+5\%$ CO_2	− 8·6	19·1	—

Effect of phenylhydrazine on respiration. If the figures for Q_{O_2} shown in Tables I–III are examined, it will be seen that the rise in glycolysis is not accompanied by a corresponding fall in respiration. Occasionally in short experiments the latter appears to be slightly increased; a similar result was observed by Warburg [1926] in ethyl *iso*cyanide poisoning of surviving tissues, but there does not seem to be any explanation at present for this. On the other hand, and particularly in experiments of greater duration, there is usually a small fall of respiration in the phenylhydrazine tissue; the extent of the fall varies, though not entirely regularly, with different tissues and is fairly uniformly found in extended experiments with testis, sometimes with some of the embryonic tissues, and is less pronounced, or absent, with Jensen sarcoma.

In considering these figures there is a suggestive parallelism with the respiration of the same tissues in the absence of glucose from the medium; this effect of glucose-lack has been fully examined in a previous publication [Dickens and Greville, 1933, 1]. It was shown that, in experiments extending over some hours, the respiration of testis falls slowly, but that of most of the other tissues used in Tables I–III is less affected by withdrawal of glucose; probably even with different samples of any particular tissue the extent of the fall is to some extent dependent on the state of nutrition of the animal. The respiration of the Jensen sarcoma is practically identical in extent whether glucose is present or not.

This suggested that the action of phenylhydrazine in inhibiting the Pasteur reaction might be connected with a loss of ability of the poisoned tissue to oxidise glucose. This question has been attacked in two ways. Firstly, the value of R.Q. in a few examples of the poisoned tissue was examined. Unfortunately little help could be expected from these measurements, for with the tissues available the fall in R.Q. in absence of glucose is by no means marked and may even be lacking entirely, as was shown by Dickens and Greville [1933, 1] in a number of instances. The most favourable example from this point of view was testis, and here the values of R.Q. and respiration both suffered a fall in the longer experiments (Table II) suggesting that carbohydrate oxidation may be reduced.

Similar measurements with Jensen sarcoma showed that neither the R.Q. nor the respiration undergoes any marked change in the poisoned tissue (Table III); here again the result is comparable with the absence of change of these quantities with the untreated tissue in glucose-free Ringer, as has been previously shown.

The most serviceable examples of cells suitable for the demonstration of carbohydrate oxidation are provided by brain grey-matter [Loebel, 1925] and the yeast-cell [Meyerhof, 1925]. In both of these, unlike the tissues already considered, the continuance of the respiration is dependent on the supply of carbohydrate from without.

The following experiments were designed to determine to what extent the oxidation of glucose is influenced in these cells by treatment with phenylhydrazine, since this appeared to be a critical test of how far it is the oxidation of carbohydrate that is specifically interfered with by this reagent.

Table V. *Rat cerebral cortex.*

		Control				Treated phenylhydrazine						
						Pre-treatment						
No.	Exp. time mins.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	R.Q.	Conc. M/lit.	Time mins.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	R.Q.	Method
1	30	11·1	3·5	—	—	5×10^{-4}	15	12·9	5·6	—	—	B
						10^{-2}	15	3·9	21·4	19·4	—	B
2	40	13·4	2·5	28·0	—	$2·5 \times 10^{-3}$	15	10·3	11·3	24·4	—	—
	90	—	—	—	—			8·6	8·5	—	—	B
	90	—	—	—	—	$2·5 \times 10^{-3}$	15	11·6	10·7	24·0	—	B*
						5×10^{-3}	15	4·4	15·7	—	—	B*
3	60	—	—	—	—	$2·5 \times 10^{-3}$	15	12·2	10·0	27·0	—	—
	120	—	—	—	—			9·6	8·8	—	—	B
	130	—	—	—	—	$2·5 \times 10^{-3}$	15	6·4	11·2	—	0·98	B†
						$2·5 \times 10^{-3}$	15	8·3	—	—	0·96	Phosphate
								5·9	—	—	1·02	Phosphate
4	60	12·3	4·3	15·9	—	$2·5 \times 10^{-3}$	15	9·5	7·0	17·8	—	B
	90	10·7	—	—	—	$2·5 \times 10^{-3}$	15	8·4	—	—	—	Phosphate
	120	12·6	2·3	—	0·98	$2·5 \times 10^{-3}$	15	9·9	7·5	17·0	1·01	B†
5	120	—	—	—	—	$2·5 \times 10^{-3}$	15	5·0	15·2	18·4	—	B†
								5·8	11·1	18·4	—	B†

Abbreviations: B = method of Warburg [1924]; B* = method of Dickens and Šimer [1931, 1]; B† = method of Dickens and Greville [1933, 3]; Phosphate = method of Dickens and Šimer [1930, 1].

Rat cerebral cortex. The experiments on rat brain tissue were carried out just as for the other tissues in Tables I–III. As in the other experiments glucose was present throughout in the media, a necessary precaution here since the respiration of brain tissue quickly suffers in absence of sugar. The results are shown in Table V. As before, it will be seen that the phenylhydrazine pre-treatment is without effect on the anaerobic glycolysis, whilst in some experiments the aerobic glycolysis rises considerably. In all of these, however, the respiration sinks, more or less slowly, and in spite of repeated trials it has not been found possible by action of phenylhydrazine with this tissue to raise the glycolysis to the anaerobic level without the occurrence of a serious fall in respiration. The R.Q. does not appear to be affected by phenylhydrazine pre-treatment of brain, and it is often similarly unaffected by lack of glucose [Dickens and Greville, 1933, 1].

Thus with brain tissue poisoned by phenylhydrazine, as the aerobic glycolysis increases there appears to be a progressive change in the respiratory metabolism towards that shown by the same tissue in absence of glucose. The question therefore arises: is the oxidation of other metabolites similarly affected? Loebel [1925] showed that the respiration of normal brain tissue can be supported

by glucose, lactate or pyruvate. In the following experiment the effect of these metabolites on the respiration of normal and poisoned brain was studied.

Rat cerebral cortex. Control brain. As control, slices prepared from two brains were treated with phenylhydrazine ($2{\cdot}5 \times 10^{-3} M$) for a time insufficient for the metabolism to be affected.

Medium	Substrate	Respiration (Q_{O_2}) 1st hr.	2nd hr.	R.Q.
Phosphate-Ringer	None added	− 8·0	− 2·6	—
	Glucose 0·2%	−11·0	− 9·6	1·03
	dl-Lactate 0·2%	−12·0	−12·0	1·03
	Pyruvate 0·2%	− 9·5	− 9·3	1·30

Bicarbonate-glucose-Ringer: Q_{O_2} − 11·2; $Q_M^{O_2}$ +5·3; $Q_M^{N_2}$ +21·5.

Phenylhydrazine brain. Pre-treatment: oxygenation for 15 minutes at room temperature with $2{\cdot}5 \times 10^{-3} M$ phenylhydrazine.

Medium	Substrate	Respiration (Q_{O_2}) 1st hr.	2nd hr.	3rd hr.	R.Q.
Phosphate-Ringer	None added	− 5·5	−2·1	−1·0	—
	Glucose 0·2%	−10·0	−4·0	−0·8	0·99
	dl-Lactate 0·2%	− 8·3	−6·0	−4·0	1·01
	Pyruvate 0·2%	−10·7	−9·6	−8·6	1·32

Bicarbonate-glucose-Ringer (1st 40 mins.): Q_{O_2} − 11·8; $Q_M^{O_2}$ +10·3; $Q_M^{N_2}$ +20·0.

These figures show how the respiration changes with time, and from them it is clear that whilst the ability to oxidise glucose and lactate is severely damaged in the partially poisoned brain, the ability to oxidise pyruvate is in no way affected. This result is particularly noteworthy, since it was shown for tumour tissue [Dickens and Šimer, 1930, 2] that whilst glucose and lactate have little effect on the level of R.Q., pyruvate increases this very markedly to above unity (Jensen sarcoma, Rous sarcoma). Except for the fact that the tumour respiration in absence of added substrate does not fall, a property known to be characteristic of brain tissue, the behaviours towards these metabolites of the partially poisoned normal tissue after treatment with phenylhydrazine and of the tumour tissue appear to be very similar or identical. Pyruvate is the only one of these three substances to be readily oxidised by the two types of tissue, in both of which the respiration is damaged in such a way that it is unable to exercise its normal influence on the aerobic glycolysis.

Baker's yeast. The behaviour towards glucose of the respiration of the yeast cells used (Table VI) was found to be essentially similar to that of brain tissue, but the fall of respiration in absence of glucose (about 90 %) in the washed cells showed that the yeast cells were even more dependent than brain on glucose from the medium for their respiration.

Phenylhydrazine pre-treatment of this yeast inhibited its respiration without appreciably affecting its power of anaerobic fermentation. The aerobic fermentation rose, and calculation of the Meyerhof quotient shows that there was only a slight inhibition of the Pasteur reaction, or in other words the poisoning of the carbohydrate respiration ran almost parallel with the appearance of the carbohydrate fermentation. Finally, with the high concentration of phenylhydrazine, poisoning of the respiration was complete, within the limits of error, whilst the fermentation in air became equal to anaerobic (HCN-poisoned) fermentation of the untreated yeast (Table VI).

As with higher cells, this toxic action of phenylhydrazine on the yeast cell is dependent on the presence of oxygen. With yeast the effect is very marked:

Table VI. *Baker's yeast.*

1. Effect of glucose-lack.

Suspension in $M/20$ NaH_2PO_4, oxygenated by bubbling the gas. 0·6 mg. dry wt. of yeast per ml.

Control portion washed three times at the centrifuge with solution of NaH_2PO_4 ($M/20$), glucose, $M/18$.

"Glucose-free" portion washed three times at the centrifuge with solution of NaH_2PO_4 ($M/20$), without glucose.

Both portions made up to suitable volume in the same solutions as used for washing.

	Q_{O_2} (Respiration)	$Q_G^{O_2}$ (Aerobic ferm.)	$Q_G^{N_2}$ (Anaerobic ferm.)
Result: (temp. 25°) Control portion with glucose	54·5	8·5	111
"Glucose-free" portion in glucose-free phosphate	{4 / 7·5	{1·5 / 7·5	4·5
"Glucose-free" portion on addition of glucose	55·5	10	123

2. Effect of treatment with phenylhydrazine.

Suspension as above of a different batch of yeast from the same commercial source. 0·3 mg. dry wt. of yeast per ml. in glucose-phosphate solution.

Control portion. Washed three times at centrifuge with glucose-phosphate solution. Made up to original volume.

Phenylhydrazine pre-treatment. To the oxygenated yeast suspension at room temperature, sufficient of a neutralised solution of phenylhydrazine hydrochloride to give the concentration stated was added. After 15 mins. centrifuged, washed three times with glucose-phosphate solution, and made up to the original volume.

Result: (temp. 25°):

Concentration of phenylhydrazine M/lit.	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$
0 (control)	80	23	180
10^{-4}	82	16	—
$2{\cdot}5 \times 10^{-4}$	68	60	167
10^{-3}	51	91	161
2×10^{-3}	10	145	150
3×10^{-3}	0	163	168
7×10^{-3}	0	175	165

Note. The values of "anaerobic fermentation" were obtained by poisoning with 2×10^{-3} N HCN.

mixture of the phenylhydrazine solution with the non-aerated suspension of yeast merely in contact with air but not shaken is not enough to cause the full alteration of metabolism. Saturation with oxygen however quickly leads to the usual result:

Baker's yeast. 1 mg. (dry wt.) per 3 ml. in NaH_2PO_4 $M/20$, glucose $M/18$; 25°. Pre-treatment with concentration of phenylhydrazine stated for 15 minutes.

		Phenylhydrazine pre-treatment						
		Nitrogen (with $CrCl_2$)		Air (not shaken)			Oxygen (shaken)	
Conc. of phenylhydrazine	Control	7×10^{-3}	10^{-3}	7×10^{-3}	3×10^{-3}	10^{-3}	7×10^{-3}	$3 \times 10^{-3} M.$
Respiration (Q_{O_2})	− 45	−47·5	−46·5	−29·5	−42·5	−47	0	− 6·5
Aerobic fermentation ($Q_G^{O_2}$)	+ 8	+17	+ 6	+39	+24	+ 9	+101	+110
Anaerobic fermentation ($Q_G^{N_2}$)	+108	—	—	—	—	—	+ 93	+109

Other reagents: phenylhydroxylamine and amyl nitrite, both of which lead to methaemoglobin formation in the erythrocyte [Heubner, 1913; Lipschitz, 1924; Warburg *et al.* 1930; 1931] and to increased respiration of the latter in glucose, are without similar effect to that of phenylhydrazine on the Pasteur reaction

Table VII. *Other reagents.*

		Control			Treated tissue				
					Conc. in Ringer				
		Q_{O_2}	$Q^{O_2}_M$	$Q^{N_2}_M$	*M*/lit.	Q_{O_2}	$Q^{O_2}_M$	$Q^{N_2}_M$	Method
1. Rat testis.	Phenylhydroxylamine†	10·0	3·5	8·8	6×10^{-4}	1·3	0·7	9·2, 7·8	B
					$1{\cdot}2 \times 10^{-4}$	7·7	2·6	—	B
	Quinone†	—	—	—	10^{-3}	4·9	1·9	3·1	B
					5×10^{-4}	4·4	1·3	3·3	B
						3·4	—	—	Phosphate
2. Jensen sarcoma.	Phenylhydroxylamine*	16·4	22·8	34·4	10^{-3}	3·9	15·0	30·0	B
	Amyl nitrite†	7·8	19·7	30·4	*ca.* *M*/100	4·2	15·2	24·6	B

Abbreviations: B = method of Warburg [1924]; Phosphate = method of Dickens and Šimer [1930, 1].
* = pre-treatment; † = in vessel with the tissue.

(Table VII), their action being to lessen the aerobic metabolism generally; in depressing Q_{O_2} they are more like the indifferent narcotics. Phenylhydroxlamine was the pure colourless recrystallised substance freshly prepared according to Wohl [1894]. It was dissolved in the Ringer solution in the concentration stated immediately before use; the solution however very quickly undergoes decomposition. Amyl nitrite (B.D.H., A.R.) was also added to the Ringer solution directly and the mixture shaken mechanically for half an hour before use. In both cases corrections for spontaneously occurring pressure changes were applied.

Since these reagents, in their reactions with haemoglobin at any rate, are without effect on the globin, whilst phenylhydrazine brings about its denaturation, it seems likely that it is to this property of protein denaturation that phenylhydrazine owes its action on the Pasteur reaction.

The mechanism of the Pasteur reaction. According to Warburg's conception [see Warburg, 1930], the increase of aerobic glycolysis without a corresponding fall in the respiration is the criterion by which we may judge the specific inhibition of the Pasteur reaction. The extent of the inhibition is to be estimated by the fall in the value of the Meyerhof quotient: (aerobic *minus* aerobic glycolysis)/(respiration). From a normal value of about 2, this quotient will sink to zero when inhibition of the Pasteur reaction is complete.

Judged by this standard, phenylhydrazine is a specific, irreversible inhibitor of the Pasteur reaction only in certain tissues; these are the tissues whose continued respiration is not dependent upon their being provided with carbohydrate from without. On the other hand, in tissues whose respiration is dependent on exogenous carbohydrate, phenylhydrazine acts as a specific poison for the respiratory enzymes, where its effect on the respiration may, as with the yeast cell, resemble that of cyanide in its completeness.

A number of questions are at once suggested by this apparently dual rôle of phenylhydrazine as a poisoner on the one hand of the Pasteur reaction and on the other of the oxidation of carbohydrate. In the first place it does not seem probable that this property possessed by phenylhydrazine of interference with glucose oxidation without interfering with the mechanism of glucose cleavage is limited to tissues whose respiration falls in the absence of glucose. It is therefore a probable assumption, supported by the evidence as far as this is available, that the mechanism of phenylhydrazine poisoning is similar in all the tissues studied,

and that the inhibition of the Pasteur reaction by phenylhydrazine in these tissues is bound up with the inhibition of glucose oxidation. The question at once arises as to whether the continuance of the Pasteur reaction is necessarily linked with oxidation of carbohydrate, or whether the oxidation of other foodstuffs may also bring about the disappearance of glycolysis. This question cannot be regarded as settled at the present time. There are very few cases where the relative amount of carbohydrate oxidised can be assessed with anything approaching the certainty with which an estimate can be formed for tissues such as brain and retina whose continued respiration depends on such supplies. Moreover, peripheral frog-nerve [Gerard, 1932] provides an example where the existence of such a relationship seems doubtful on the basis of existing information; it should be remembered however that this tissue presents metabolic peculiarities, such as the delayed appearance of glycolysis in nitrogen [Gerard and Meyerhof, 1927] not found in other cases [see also Chang and Gerard, 1933]. Again, with sugars other than glucose (*e.g.* fructose), oxidation of carbohydrate may proceed without anaerobic lactic acid formation (brain, see Loebel [1925], brain and retina, see Dickens and Greville [1933, 1]); it cannot however be assumed that oxidation of glucose and fructose by tissues necessarily proceeds along similar routes, and it is therefore permissible to limit the present discussion to the rôle of the oxidation of glucose in the Pasteur mechanism.

As far as the glucose metabolism of the mammalian tissues here described is concerned, the evidence for the hypothesis advanced by Dickens and Šimer [1930, 2], that the Meyerhof cycle is connected mainly or wholly with the oxidation of carbohydrate, rests principally on observations of R.Q. [Dickens and Šimer, 1930, 2; 1931, 2; Dickens and Greville, 1933, 1, 3], on the effect of sugar-lack on the ammonia formation [Dickens and Greville, 1933, 2] and on the respiration and R.Q. [Dickens and Greville, 1933, 1].

If the estimate of sugar oxidation derived from these values be accepted, it is found that in all the normal tissues studied glucose oxidation runs parallel with their ability to form lactic acid from glucose. The criticism which may be applied to this conclusion is that the evidence of carbohydrate oxidation is necessarily indirect in most cases. Though there does not appear to be any reason to doubt the correctness of the estimates of carbohydrate oxidation, some more direct demonstration of a link between carbohydrate oxidation and cleavage is certainly desirable. Phenylhydrazine may be regarded as helping to fill this gap, but the question cannot yet be regarded as answered. Do other inhibitors of the Pasteur reaction behave towards carbohydrate oxidation similarly to phenylhydrazine, or is the inhibition of carbohydrate oxidation merely an incidental property possessed by phenylhydrazine? Unfortunately Warburg's [1926] experience with ethyl *iso*cyanide does not help us to decide this question, since he did not make use of any tissue in which the respiration falls appreciably in absence of glucose in short experiments.

With yeast cells Warburg states that "similar though not equally convincing experiments can be carried out with yeast cells.... Without the respiration of the yeast being obstructed to any considerable extent the aerobic fermentation is intensified in the presence of 10^{-3} *N* ethyl *iso*cyanide. The aerobic fermentation by yeast in 10^{-3} *N* ethyl *iso*cyanide does not however attain the dimension of the anaerobic fermentation." As this experiment would provide a crucial test of the interdependence or otherwise of the fermentation and carbohydrate oxidation in this case, it is proposed to repeat the observations made with phenylhydrazine using ethyl *iso*cyanide and other similar reagents in this and related examples when opportunity occurs.

SUMMARY.

The action of phenylhydrazine and some allied substances on the metabolism of surviving tissues has been investigated. Phenylhydrazine, in low concentration, is without action on the anaerobic glycolysis of animal tissues or on the anaerobic fermentation of yeast. On the other hand, in the presence of oxygen, it causes the aerobic lactic acid formation of a wide range of animal tissues and the aerobic fermentation of baker's yeast to rise to a value near or equal to that of the same cells anaerobically. The effect of the same treatment on the respiration is described, and it is found that the tissues which are dependent on the oxidation of glucose (or lactate) for their continued respiration undergo a progressive fall of respiration after treatment. Baker's yeast also belongs to this class of cell. However, the majority of animal tissues are not so dependent, and their respiration is much less obviously affected by phenylhydrazine. Tumour tissue (Jensen sarcoma) behaves similarly to this group of normal cells, and its already high aerobic glycolysis is caused by this treatment to rise to the anaerobic value. The bearing of these observations on the nature of the Pasteur reaction is discussed.

REFERENCES.

Barron (1930). *J. Exp. Med.* **52**, 447.
Bumm and Appel (1932). *Z. physiol. Chem.* **210**, 79.
Chang and Gerard (1933). *Amer. Chem. J.* **104**, 291.
Dickens and Greville (1933, 1). *Biochem. J.* **27**, 832.
—— —— (1933, 2). *Biochem. J.* **27**, 1123.
—— —— (1933, 3). *Biochem. J.* **27**, 1479.
—— and Šimer (1930, 1). *Biochem. J.* **24**, 905.
—— —— (1930, 2). *Biochem. J.* **24**, 1301.
—— —— (1931, 1). *Biochem. J.* **25**, 973.
—— —— (1931, 2). *Biochem. J.* **25**, 985.
Friedemann and Kendall (1929). *J. Biol. Chem.* **82**, 23.
Gerard (1932). *Physiol. Rev.* **12**, 472 (p. 496).
—— and Meyerhof (1927). *Biochem. Z.* **191**, 125.
Heubner (1913). *Arch. exp. Path. Pharm.* **72**, 241.
Krah (1930). *Biochem. Z.* **219**, 432.
Lipschitz (1924). *Ergebn. Physiol.* **23**, 1.
Loebel (1925). *Biochem. Z.* **161**, 219.
Meyerhof (1925). *Biochem. Z.* **162**, 43.
Negelein (1925). *Biochem. Z.* **165**, 122.
Warburg (1924). *Biochem. Z.* **152**, 51.
—— (1926). *Biochem. Z.* **172**, 432.
—— (1929). *Biochem. Z.* **204**, 482.
—— (1930). Preface to Metabolism of tumours. (Constable, London.)
—— Kubowitz and Christian (1930). *Biochem. Z.* **227**, 245.
—— —— —— (1931). *Biochem. Z.* **242**, 170.
—— Posener and Negelein (1924). *Biochem. Z.* **152**, 309.
Wohl (1894). *Ber. deutsch. chem. Ges.* **27**, 1432.

LXXVIII. THE HYDROLYSIS OF LECITHO-VITELLIN BY PEPSIN AND BY TRYPSIN-KINASE.

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It has long been recognised that egg-yolk and milk contain somewhat similar proteins. In particular the two phosphoproteins, caseinogen and lecitho-vitellin, show a certain similarity in constitution and properties, though the latter is peculiar in respect of its content of iron and copper. For example Rimington and Kay [1926] found that hydrolysis of caseinogen with 1 % sodium hydroxide liberated all the organically bound phosphorus of the protein as inorganic phosphate. McFarlane [1932], working with lecitho-vitellin, showed that the action of 1 % alkali resulted in the formation of an acid-insoluble material containing 50 % of the original nitrogen, only a trace of phosphorus and no iron, while the acid-soluble products were rich in both iron and phosphorus.

Little information is available with regard to the action of enzymes on lecitho-vitellin. Miescher [1870] observed that an insoluble material separated out from a peptic digest of egg-yolk. This material was named "haematogen" by Bunge [1885], who found that it contained iron in organic combination. The nature of this insoluble product was studied in greater detail by Hugounenq and Morel [1905, 1, 2; 1906] and later by Posternak [1927], whose results indicated that the so-called "haematogen" might not be a pure compound. These investigations were however carried out before the separate preparation of livetin, the second protein of egg-yolk, by Kay and Marshall [1928; see also Plimmer 1908].

The enzymic cleavage of caseinogen has, on the other hand, been extensively investigated. The studies of Rimington [1927, 1, 2], Rimington and Kay [1926] and Holter *et al.* [1932], on the products of enzymic hydrolysis of caseinogen, have thrown considerable light on the structure of the caseinogen molecule. The nature of the enzymic hydrolysis has been further studied by Stirling and Wishart [1932], who observed certain well-defined differences in the digestion of caseinogen by pepsin and by trypsin-kinase. Briefly summarised these differences were as follows:

1. In tryptic digests acid-soluble phosphorus was liberated more rapidly than acid-soluble nitrogen; the reverse held for pepsin.

2. In peptic digests a stage was reached early in digestion (presumably by the liberation of an acid-soluble material containing no phosphorus) in which the residual acid-insoluble material had a N/P ratio of 12·8. Further, the acid-insoluble residue preserved approximately this ratio throughout a prolonged

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period even though it was considerably diminished in amount by further hydrolysis. In the later stages of digestion, however, the N/P ratio fell slowly as a result of the liberation of a nitrogen-rich material. It was suggested that the final acid-insoluble residue might be identical with Linderstrøm-Lang's [Holter *et al.*, 1932] "paranuclein," with a N/P ratio of 8.

3. With trypsin the course of the digestion was entirely different. There was a rapid preferential liberation of acid-soluble phosphorus, so that the N/P ratio of the insoluble residue rose progressively until, under suitable conditions, it reached infinity. At this stage 30 % of the nitrogen still remained acid-insoluble.

On the basis of these findings it was suggested by Stirling and Wishart that trypsin breaks down peptide linkages in proximity to the phosphorus atoms, the resultant phosphorus-containing cleavage products being of small molecular size. On the other hand, the initial peptic rupture occurs at linkages remote from the phosphorus atoms, the phosphorus-containing cleavage products being of much greater molecular dimensions and therefore insoluble in trichloroacetic acid.

It appeared from the results of these experiments that the same method of attack might be usefully employed in elucidating the structure of lecitho-vitellin.

Experimental.

Preparation of lecitho-vitellin.

The intact yolks of 24 eggs were washed in running water and finally in 0·9 % NaCl until free from egg-white. The yolk membrane was punctured and the contents, without membrane and chalazae, mixed with an equal volume of 10 % NaCl and extracted with an equal volume of ether in a mechanical shaker. Extraction was repeated until the ether was colourless and no qualitative test for lecithin could be obtained. Usually 6 or 7 thorough extractions were required, each with an amount of ether equal to the volume of the egg-yolk solution. The saline solution was poured into 20 volumes of distilled water and, after standing overnight, the supernatant liquid was siphoned off from the white protein precipitate. The precipitate was centrifuged and dissolved in 10 % NaCl; the solution was then filtered and poured into 20 volumes of water. The dissolution and precipitation were repeated, the precipitate being washed well with water before centrifuging for the last time. The protein was extracted with cold 80 % and then 97 % ethyl alcohol for 3 hours each and the residue filtered off, washed with absolute alcohol, ether and finally toluene. The resulting protein was easily friable and passed through a 60-mesh sieve.

Two such preparations of lecitho-vitellin were made, identical procedures being followed. The method given differs in two respects from those recorded in the literature: (1) the use of cold ethyl alcohol in the final lipoid extraction; and (2) the addition of toluene after ether as a dehydrating agent (recommended by Van Slyke [1923] for caseinogen). The two products were alike in appearance and general behaviour. Analysis gave the following results:

		I %	II %
	Moisture	22·42	23·9
% dry weight	Nitrogen	15·78	16·78
	Phosphorus	0·83	1·03
	Ash	2·20	2·38

These show very fair agreement with the analyses of Osborne and Campbell [1900] and Calvery and White [1930].

It is stated that pure vitellin is insoluble in water and almost insoluble in dilute alkali; vitellin in loose combination with lecithin is relatively soluble. Both the above preparations were soluble up to 1 % in approximately $M/40$ KOH. Though insoluble directly in acid, an acid solution could readily be obtained by the addition of acid to the original alkaline solution. The solubility of the preparations at first suggested that they still contained considerable quantities of lecithin, but extraction of the protein with boiling ethyl alcohol showed that the lecithin content could not be more than 2 %.

Experiments were begun with preparation I but unfortunately this did not suffice for all the experiments, and for some of the later experiments the second preparation had to be used. A determination of the N/P ratio of the substrate solution was however made during each experiment, and these figures are recorded in the tables. In evaluating the changes of N/P ratio which occur during the course of each digestion, due consideration must be given to the initial value of this ratio.

Vitellin solutions. In making solutions of the protein for the enzymic hydrolyses we adopted the following method. A weighed quantity (about 2 g.) of vitellin was rubbed up to a smooth paste with a little water, further water was then added to a total volume of about 50 ml.; 2·5 ml. M KOH per g. of vitellin were then added slowly and the rubbing up continued until the preparation, which first became viscous, again liquefied. The total volume was now made up to 75 ml. per g. of vitellin and the solution centrifuged after standing overnight. The supernatant liquid was used for the digestion experiment. The solution so obtained had p_H approximately 9 and, when added to the buffer of p_H 8·8 for the trypsin experiments, did not materially alter the p_H of the latter. For the pepsin experiments a predetermined quantity of N HCl was added to the vitellin solution to bring the p_H approximately to 2 before mixing with the acid buffer. The vitellin, at first precipitated by this acid, redissolved immediately on further addition of acid. Determination of the p_H at various stages during the enzymic hydrolyses showed that it remained well within the optimum range for each enzyme.

By this method we were never able to prepare solutions of higher concentration than about 1 %. This was unfortunate since the nitrogen and phosphorus contents of the enzyme and substrate controls then formed not inconsiderable proportions of the total nitrogen and total phosphorus present. In all digestions substrate and enzyme controls were followed throughout the time course of the experiment, and the tabulated values are net values.

Enzyme solutions. Solutions of B.D.H. trypsin and B.D.H. pepsin were used. The concentration of 0·75 % was chosen after carrying out 2 experiments, one with trypsin and one with pepsin, in which a series of different concentrations of enzyme was allowed to act on the substrate for a period of 2 hours. The results of these experiments are reported in Tables III A and III B.

Buffer solutions. The buffer solutions consisted of a KCl-borate mixture of p_H 8·8 and a KCl-HCl mixture of p_H 1·8. On occasion these were employed in a concentration 4 times that given in the previous paper in order that the volume saved thereby might be utilised for additional substrate solution (see below).

Procedure.

This was identical with that described in the previous paper [Stirling and Wishart, 1932] except that the digest-tubes on occasion contained 8 ml. of substrate and 1 ml. of the more concentrated buffer instead of 5 ml. and 4 ml. respectively. A large number of experiments were carried out in which the time-

Table IA. *Trypsin-kinase (7·5 mg. in 10 ml. solution).*

Exp. no.	5	6
Total N (mg. per 10 ml. sol.)	11·61	5·68
Total P (mg. per 10 ml. sol.)	0·538	0·348

	Exp. 5				Exp. 6			
	Acid-sol. N		Acid-sol. P		Acid-sol. N		Acid-sol. P	
Time	Amount mg.	% of total	Amount mg.	% of total	Amount mg.	% of total	Amount mg.	% of total
10 mins.	0·53	4·6	0·059	11·0	0·43	7·6	0·055	15·8
20 mins.	0·97	8·3	0·095	17·7	0·69	12·1	0·084	24·1
30 mins.	1·35	11·6	0·119	22·1	0·88	15·5	0·097	27·8
40 mins.	1·58	13·6	0·126	23·4	1·13	20·0	0·111	31·8
50 mins.	1·98	17·0	0·149	27·6	1·32	23·2	0·114	32·7
60 mins.	2·17	18·7	0·173	32·1	1·50	26·4	0·093	26·6
2 hrs.	3·14	27·0	0·221	41·0	1·95	34·2	0·150	43·0
3 hrs.	3·91	33·7	0·238	44·2	2·27	39·9	0·162	46·4
4 hrs.	4·54	39·1	—	—	2·65	46·6	0·188	54·0
5 hrs.	5·08	43·8	0·255	47·2	2·73	48·0	0·187	53·6
6 hrs.	5·36	46·2	0·268	49·9	2·98	52·5	0·191	54·7
9 hrs.	6·72	57·9	0·306	57·0	3·19	56·2	0·216	62·0
12 hrs.	7·13	61·4	0·335	62·0	3·60	63·3	0·214	61·2
24 hrs.	8·39	72·3	0·376	70·0	4·62	81·3	0·239	68·5

Table IB. *Pepsin (7·5 mg. per 10 ml. solution).*

Exp. no.	8	9
Total N (mg. per 10 ml. sol.)	5·31	9·82
Total P (mg. per 10 ml. sol.)	0·237	0·460

	Exp. 8				Exp. 9			
	Acid-sol. N		Acid-sol. P		Acid-sol. N		Acid-sol. P	
Time	Amount mg.	% of total	Amount mg.	% of total	Amount mg.	% of total	Amount mg.	% of total
10 mins.	1·89	35·6	0·039	16·4	2·46	25·0	—	—
20 mins.	2·63	49·6	0·058	24·4	3·55	36·1	0·072	15·7
30 mins.	2·88	54·4	0·069	29·0	4·35	44·3	0·087	18·9
40 mins.	3·02	57·0	0·074	31·1	4·78	48·7	0·016	23·0
50 mins.	3·26	61·5	0·076	31·9	5·17	52·7	0·105	22·8
60 mins.	3·37	63·6	0·072	30·3	5·44	55·4	0·113	24·6
2 hrs.	3·44	64·9	0·065	27·3	6·28	63·9	0·123	26·7
3 hrs.	3·86	72·8	0·058	24·4	6·50	66·2	0·128	27·8
4 hrs.	3·99	75·2	0·083	34·9	6·84	69·6	0·129	28·0
5 hrs.	4·12	77·6	0·071	29·8	6·94	70·6	0·133	28·9
6 hrs.	4·21	79·4	0·063	26·5	7·15	72·8	0·131	28·5
9 hrs.	4·21	79·4	0·063	26·5	7·41	75·5	0·129	28·0
*12 hrs.	4·20	79·2	0·050	21·0	7·64	77·8	0·127	27·6
24 hrs.	4·40	82·8	0·044	18·5	6·83	69·5	0·124	27·0

* 15 hrs. in exp. no. 8.

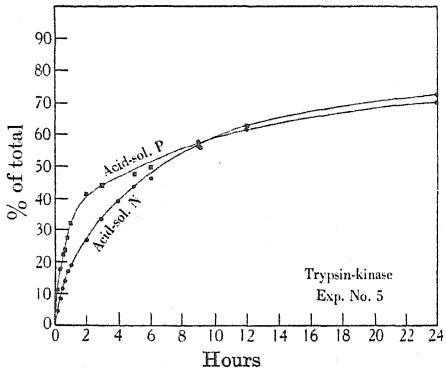


Fig. 1.

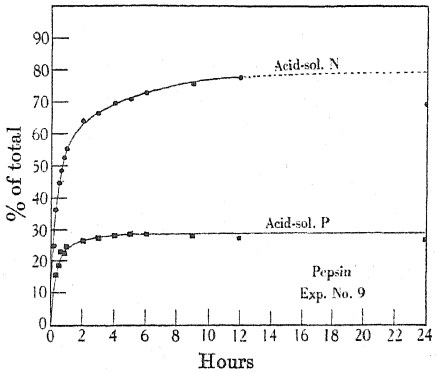


Fig. 2.

course of the digestion of various concentrations of substrate by 0·75 % enzyme was followed. Of these, 2 experiments for each enzyme are given in Tables I A (trypsin) and I B (pepsin). Graphs are also given for 1 experiment with each enzyme (Figs. 1 and 2). The nitrogen-phosphorus ratios of the acid-soluble and acid-insoluble material at each stage in these experiments are given in Tables II A and II B.

Table II A. *N : P ratios.*

Exp. no.			5		6	
N : P ratio of original substrate			21·6		16·4	
	Exp. 5			Exp. 6		
Time	Acid-sol.	Acid-insol.		Acid-sol.		Acid-insol.
10 mins.	9·1	23·2		7·9		18·0
20 mins.	10·2	24·1		8·1		19·0
30 mins.	11·3	24·6		9·1		19·2
40 mins.	12·6	24·4		10·2		19·2
50 mins.	13·2	24·8		11·5		18·7
60 mins.	12·6	25·8		16·1		16·4
2 hrs.	14·2	26·7		13·0		18·9
3 hrs.	16·5	25·7		14·0		18·4
4 hrs.	—	—		14·1		18·9
5 hrs.	19·9	23·1		14·6		18·4
6 hrs.	20·0	23·2		15·6		17·2
9 hrs.	22·0	21·1		14·8		18·8
12 hrs.	21·2	22·0		16·8		15·6
24 hrs.	22·1	19·9		19·4		9·8

Table II B. *N : P ratios.*

Exp. no.			8		9	
N : P ratio of original substrate			22·4		21·5	
	Exp. 8			Exp. 9		
Time	Acid-sol.	Acid-insol.		Acid-sol.		Acid-insol.
10 mins.	48·6	17·1		—		—
20 mins.	45·5	15·0		49·4		16·1
30 mins.	41·7	14·4		50·0		14·6
40 mins.	40·9	14·0		45·1		14·2
50 mins.	43·1	12·7		49·3		13·1
60 mins.	46·8	11·7		48·3		12·6
2 hrs.	52·8	10·8		51·0		10·5
3 hrs.	66·6	8·1		50·9		10·0
4 hrs.	48·0	8·5		52·9		9·0
5 hrs.	58·0	7·4		52·2		8·8
6 hrs.	66·8	6·3		54·5		8·1
9 hrs.	66·8	6·3		57·5		7·3
*12 hrs.	84·0	5·9		60·2		6·5

* 15 hrs. in exp. no. 8.

Tables III A and III B show the results obtained when the same substrate solution was digested for a uniform period of 2 hours with enzyme concentrations varying from 100 mg. to 1 mg. per 10 ml.

In addition to these, a third type of experiment was carried out which has no parallel in the previous paper. The production of an insoluble precipitate, analogous to that named "haematogen," by Bunge, was a striking feature of the peptic digests. We therefore carried out a series of digestions with enzyme concentrations of 100 mg. and 20 mg. of pepsin per 10 ml. in which no trichloroacetic acid was added, but the insoluble material was quickly filtered off, and the nitrogen and phosphorus contents of the filtrate were examined. Table IV A records the results of this experiment and shows the early formation of this "haematogen" material and its remarkable resistance to peptic hydrolysis. Table IV B records the nitrogen-phosphorus ratios for filtrate and residue in this experiment.

Table III A. *Effect of varying trypsin concentration.*

Total N 8·95 mg. per 10 ml.
Total P 0·579 mg. per 10 ml.
N : P ratio of original substrate 15·5
Time of incubation 2 hours.

Trypsin (mg. per 10 ml. sol.)	Acid-sol. N as % of total N	Acid-sol. P as % of total P	N : P ratio	
			Acid-sol. material	Acid-insol. material
50	82·5	61·8	20·6	7·1
25	74·5	58·2	19·8	9·4
10	56·7	40·6	21·6	11·3
5	40·7	38·2	16·5	14·8
2·5	27·2	34·5	14·4	15·9
1	15·1	19·3	12·0	16·3

Table III B. *Effect of varying pepsin concentration.*

Total N 11·10 mg. per 10 ml.
Total P 0·577 mg. per 10 ml.
N : P ratio of original substrate 19·2
Time of incubation 2 hours.

Pepsin (mg. per 10 ml. sol.)	Acid-sol. N as % of total N	Acid-sol. P as % of total P	N : P ratio	
			Acid-sol. material	Acid-insol. material
100	77·8	38·1	39·3	6·9
50	77·2	32·2	46·1	6·5
25	74·7	30·7	46·7	7·0
10	69·7	30·1	44·5	8·3
5	61·3	29·2	40·3	10·5
1	34·3	15·9	41·4	15·0

Table IV A.

Exp. no.	10	11
Total N (mg. per 10 ml.)	8·08	8·08
Total P (mg. per 10 ml.)	0·501	0·501
Enzyme conc.	100 mg.	20 mg.

Time	Composition of filtrate			
	% N	% P	% N	% P
1 hr.	86·5	25·5	80·0	19·9
2 hrs.	86·1	27·5	79·3	22·4
3 hrs.	—	—	78·5	22·8
4 hrs.	—	—	79·8	22·8
9 hrs.	—	—	82·7	25·3
24 hrs.	—	—	87·8	27·2

Table IV B. *N : P ratios of filtrate and residue.*

Exp. no.	10	11
N : P ratio of original substrate	16·1	16·1
Enzyme conc.	100 mg.	20 mg.

Time (hrs.)	N : P ratio of			
	Filtrate	Residue	Filtrate	Residue
1	54·5	2·9	64·7	4·0
2	50·3	3·1	57·1	4·3
3	—	—	55·5	4·5
4	—	—	56·3	4·25
9	—	—	52·6	3·7
24	—	—	52·1	2·7

Discussion.

There are certain points of resemblance between the experiments now reported and those recorded by Stirling and Wishart [1932], but the resemblance is not complete—a circumstance which may be ascribed to the apparently greater complexity of the lecitho-vitellin molecule.

As with caseinogen, the first step in the degradation of the protein by either enzyme does not yield products which are completely soluble in trichloroacetic acid. This is evident from the N/P ratios of the earlier stages in Tables IIA and IIB, and it confirms the criticism, made in the previous paper, concerning experiments on the kinetics of enzyme action which are based on the assumption that trichloroacetic acid precipitates only the unattacked substrate.

As with caseinogen there is a preferential liberation of acid-soluble phosphorus in tryptic digests and of acid-soluble nitrogen in peptic hydrolysis. With trypsin however the resemblance is only superficial. With the lower concentrations of caseinogen substrate, all the phosphorus was rendered acid-soluble, so that the nitrogen-phosphorus ratio of the acid-insoluble material gradually rose to infinity. In the present experiments (Table IA) the preferential liberation of phosphorus by trypsin ceases when about half of the nitrogen and phosphorus have been rendered acid-soluble, and thereafter the nitrogen liberation may even gain on that of the phosphorus. A similar condition is revealed in the results shown in Table IIIA, where the time of reaction was kept constant, and the enzyme concentration was varied. With the lower enzyme concentrations more phosphorus than nitrogen was rendered acid-soluble; with concentrations which would liberate about 40 % of the total nitrogen the percentage liberations of nitrogen and phosphorus were equal; while, with the stronger enzyme concentrations, there was a relatively greater liberation of acid-soluble nitrogen.

It is also noteworthy that in none of the experiments with lecitho-vitellin, however prolonged the digestion, or however strong the enzyme concentration, have we been able to secure a liberation of more than about 80 % of the total nitrogen and 70 % of the total phosphorus of the substrate.

The results of Stirling and Wishart [1932] on caseinogen were explicable on the assumption that the phosphorus of caseinogen was concentrated in one relatively small group. The results of the time-course experiments on lecitho-vitellin, together with the maxima of hydrolysis, suggest that such a hypothesis will not hold for lecitho-vitellin, and that there must be at least two phosphoric complexes in the molecule, one having similar properties to that of caseinogen and the other being highly resistant to tryptic hydrolysis.

Turning to the peptic digestions, Tables IB and IIIB show that there is a preferential liberation of acid-soluble nitrogen. In Tables IVA and IVB it may be seen that, with an enzyme concentration of 20 mg., the shorter periods of digestion result in the formation of an insoluble material or "haematogen" embodying about 20 % of the original nitrogen and 77 % of the original phosphorus, thus giving a N/P ratio of just over 4. This "haematogen" is obviously resistant to further peptic hydrolysis since it remains unchanged for at least 4 hours in the presence of the weaker enzyme concentration. Indeed, it is only at the ninth and twenty-fourth hours at this enzyme concentration that further degradation of the "haematogen" is observed. The change is however slight and, as Table IVA shows, affects the nitrogen slightly more than the phosphorus. With the stronger enzyme concentration degradation is more rapid.

Turning now to Exps. 8 and 9 (Tables IB and IIB), in which the cleavage products were separated by trichloroacetic acid, it appears that "haematogen"

is acid-insoluble—a fact which we have confirmed directly—and that digestion is confined mainly to the non-haematogen portion, proceeding towards the attainment of a N/P ratio in the acid-insoluble material approximating to that of the insoluble material of Tables IVA and B. (The ratios are not identical, but due weight must be given to the fact that in Table IVB the N/P ratio of the original substrate is 16, while in Table IIB, it approximates to 21.)

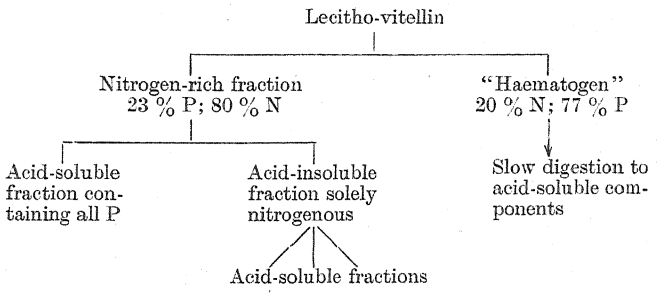
In the digestion of the non-haematogen portion the liberation of the phosphorus of this fraction in acid-soluble form is more rapid than that of the nitrogen of the same fraction. This can be brought out by expressing the amounts of acid-soluble nitrogen and phosphorus, say in Exp. 9 (Table IB), not as percentages of the total nitrogen and phosphorus, but as percentages of the non-haematogen nitrogen and phosphorus, these being taken as 7·64 mg. and 0·13 mg. respectively. The results are as follows:

Experiment No. 9. To show relative rates of liberation of phosphorus and nitrogen from the non-haematogen portion.

Total non-haematogen-nitrogen assumed to be 7·64 mg.
Total non-haematogen-phosphorus assumed to be 0·130 mg.

Time	Acid-sol. N as % of total non-haematogen-N	Acid-sol. P as % of total non-haematogen-P
10 mins.	32·2	—
20 mins.	46·4	55·5
30 mins.	56·8	67·0
40 mins.	62·6	81·6
50 mins.	67·6	80·8
60 mins.	71·1	87·0
2 hrs.	82·0	94·8
3 hrs.	85·1	98·5
4 hrs.	89·8	99·1
5 hrs.	91·0	104·0
6 hrs.	93·6	101·0
9 hrs.	97·0	—
12 hrs.	100·0	—
24 hrs.	89·2	—

These observations seem to allow us to envisage at least 4 stages in the peptic digestion of lecitho-vitellin, which, although occurring for the most part simultaneously, may be thus separately described. First, a cleavage of the protein molecule into 2 fractions, one containing 80 % of the original nitrogen and 23 % of the phosphorus, and the other being "haematogen." Second, in the nitrogen-rich fraction, disruption of peptide linkages close to its phosphorus group leads to the rapid liberation of all this phosphorus in acid-soluble form, leaving a residue which is entirely nitrogenous and still of sufficient complexity to be acid-insoluble. The third stage is the disruption of this nitrogenous



residue into acid-soluble fractions. Finally there is the very slow and feeble digestion of the "haematogen" portion of which we have but slight evidence. This series of reactions is shown concisely in the diagram (p. 557).

It is evident that such a conception of the process of peptic digestion requires the presence of at least two widely separated or dissimilar phosphoric complexes in the vitellin molecule. This assumption is in accordance with the observations on tryptic digestion already discussed.

In this connection it is interesting to note that in tryptic digestion only 70 % of the protein-phosphorus could be rendered acid-soluble, whilst in peptic digestion a somewhat similar amount (77 %) of the phosphorus remained acid-insoluble as the "haematogen" fraction and was highly resistant to further peptic digestion. One might be tempted to assume identity between these fractions. Such an assumption seems however to be unwarranted in view of Posternak's [1927] preparation from the digestion of the residue from ether extraction of egg-yolk of a fraction rich in both phosphorus and iron, which is highly resistant to both peptic and tryptic actions.

Summary.

The action of trypsin-kinase and pepsin on lecitho-vitellin has been studied. Products are formed which are not completely soluble in trichloroacetic acid. With pepsin the rate of liberation of acid-soluble nitrogen is greater than that of acid-soluble phosphorus, and the production of "haematogen" with a N/P ratio of 4·25 is a striking feature of these digests.

With trypsin the rate of liberation of acid-soluble phosphorus relative to that of acid-soluble nitrogen has been shown to depend on the enzyme concentration employed. The maximum of hydrolysis is reached, however, at all concentrations at 80 % of the total nitrogen and 70 % of the total phosphorus.

It is considered that the vitellin molecule must contain at least two widely separated or dissimilar phosphoric complexes, one being highly resistant to enzymic attack.

REFERENCES.

Bunge (1885). *Z. physiol. Chem.* **9**, 49.
Calvery and White (1930). *J. Biol. Chem.* **94**, 635.
Holter, Linderstrøm-Lang and Funder (1932). *Z. physiol. Chem.* **260**, 85.
Hugounenq and Morel (1905, 1). *Compt. Rend. Acad. Sci.* **140**, 1065.
—— (1905, 2). *Compt. Rend. Acad. Sci.* **141**, 848.
—— (1906). *Compt. Rend. Acad. Sci.* **142**, 805.
Kay and Marshall (1928). *Biochem. J.* **22**, 1264.
Miescher (1870). *Med.-chem. Untersuchungen*, **4**, 502.
McFarlane (1932). *Biochem. J.* **26**, 1061.
Osborne and Campbell (1900). *J. Amer. Chem. Soc.* **22**, 413.
Plimmer (1908). *J. Chem. Soc.* **93**, 1500.
Posternak (1927). *Compt. Rend. Acad. Sci.* **184**, 909.
Rimington (1927, 1). *Biochem. J.* **21**, 204.
—— (1927, 2). *Biochem. J.* **21**, 1179, 1187.
Rimington and Kay (1926). *Biochem. J.* **20**, 777.
Stirling and Wishart (1932). *Biochem. J.* **26**, 1989.
Van Slyke (1923). *Proc. World's Dairy Congress*, **2**, 1145.

LXXIX. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXXVII. (*a*) ON THE PRODUCTION OF HYDROXYANTHRAQUINONES BY SPECIES OF *HELMINTHOSPORIUM*. (*b*) ISOLATION OF TRITISPORIN, A NEW METABOLIC PRODUCT OF *HELMINTHOSPORIUM TRITICI-VULGARIS* NISIKADO. (*c*) THE MOLECULAR CONSTITUTION OF CATENARIN.

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IT has been previously shown [Charles *et al.*, 1933] that the mycelium of *Helminthosporium gramineum* Rabenhorst, when grown on a synthetic medium containing glucose as the sole source of carbon, contains surprisingly large amounts (30 %) of hydroxyanthraquinones, consisting of a mixture of helminthosporin, $C_{15}H_{10}O_5$ (I), and hydroxy*iso*helminthosporin, $C_{15}H_{10}O_6$ (III), approximately in the proportion of two to three parts of the former to one part of the latter. Further, we have shown [1933, 1] that the mycelium of *H. cynodontis* Marignoni and *H. euchlaenae* Zimmermann, when grown under the same conditions, contains in each case a third new hydroxyanthraquinone, cynodontin, $C_{15}H_{10}O_6$ (II). This hydroxyanthraquinone is the only one produced by *H. euchlaenae*, but it is accompanied in the case of *H. cynodontis* by small amounts of helminthosporin (I).

These observations have now been extended to many different species and strains of *Helminthosporium*, about 40 in all, most of which were obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland. While this further work has resulted in the discovery of only one hitherto undescribed hydroxyanthraquinone, it has led to a number of interesting observations which are recorded in this paper; numbers of the species examined gave no trace of hydroxyanthraquinones, but some of them gave considerable yields of one or more of the above-mentioned hydroxyanthraquinones.

The procedure followed was to grow the chosen species on a Czapek-Dox solution, filter off and dry the mycelium and extract it with a suitable solvent, *e.g.* chloroform or acetone. Under these conditions the results described have been obtained with the following species:

(*a*) *H. catenarium* Drechsler. Considerable yields of hydroxy*iso*helminthosporin (III) in an almost pure condition were isolated, and hence it is proposed to change the name of this hydroxyanthraquinone from that previously given

it to *catenarin* (III). *H. catenarium* was originally described by Drechsler [1923], who isolated it from wood reed grass (*Cinna arundinacea* L.) occurring near Brookline, New York, on the northern coast of Long Island. Drechsler gave the name *H. catenarium* to the species "because of its tendency toward the formation of chains of spores in nature as well as in pure culture."

(*b*) *H. tritici-vulgaris* Nisikado. Considerable yields of catenarin (III), together with smaller amounts of the new metabolic product *tritisporin* (see below) and helminthosporin (I) were isolated. Nisikado [1929, 1, 2] isolated and described *H. tritici-vulgaris* from diseased wheat (*Triticum vulgare* Vill.). This organism produces on wheat leaves a yellow spot disease which is of fairly common occurrence in Japan.

(*c*) *H. velutinum* Link. The only hydroxyanthraquinone isolated from this species was catenarin (III), but the mycelium also contained appreciable amounts of ergosterol. *H. velutinum* Link was first described by Link as long ago as 1809, and was found by him to be parasitic upon beech wood.

(*d*) *H. avenae* Eidam. Cynodontin (II) was isolated in small amounts and in almost pure condition. It was accompanied by small amounts of ergosterol. *H. avenae* was described originally by Eidam [1891], who found this organism on, and causing leaf spot disease of, oats occurring in Silesia. The disease on oats caused by *H. avenae* is widely distributed, having been recorded from almost all parts of the world.

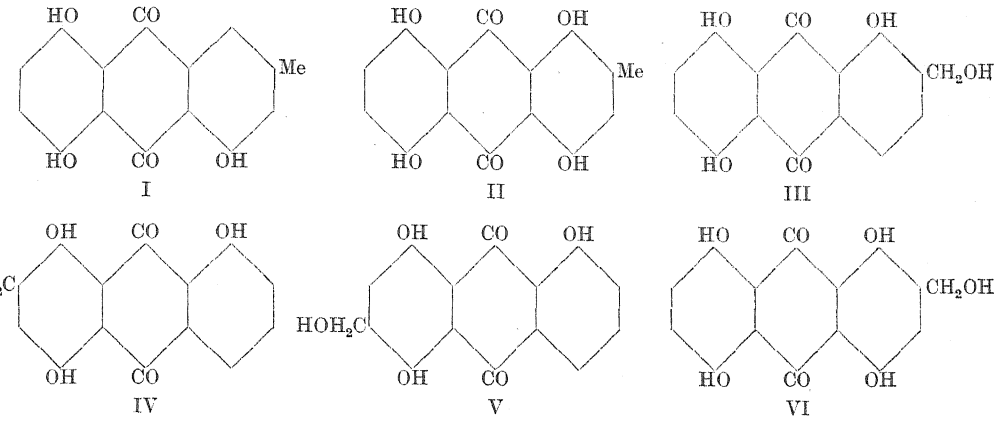
Definite evidence was obtained by colour reactions of the production of hydroxyanthraquinones, although in amounts too small for isolation and identification, by the following species of *Helminthosporium*, which are arranged approximately in the order of decreasing intensity of the colour reactions given: *H. brizae* Nisikado, *H. pedicellatum* Henry, *H. geniculatum* Tracy and Earle, *H. leptochloae* Nisikado and Mke, *H. teres* Sacc., *H. filicicola* P. Henn, *H. torulosum* (Syd.) Ashby, *H. coicis* Nisikado, *H. gibberosporum* Curzi and *H. Yamadai* Nisikado.

No hydroxyanthraquinones could be detected by colour reactions from any of the following species: *H. setariae* Sawada, *H. interseminatum* Berk. and Rav., *H. anomalum* Abbott, *H. monoceras* Drechsler, *H. sacchari* Butler, *H. sativum* P.K. and B., *H. nodulosum* (B. and C.) Sacc., *H. hurae* P. Henn, *H. oryzae* Br. de Haan, *H. turcicum* Passer, *H. Kusanoi* Nisikado, *H. Leersii* Atkinson, *H. maydis* Nisikado and Mke, *H. panici-miliacei* Nisikado, *H. Ravenelii* Curtis and *H. zizaniae* Nisikado.

The results obtained point to an interesting correlation between the biochemical synthesis of hydroxyanthraquinones by species of *Helminthosporium* and their morphological characteristics. Nisikado [1929, 2] divides the genus *Helminthosporium* into two sub-genera on morphological grounds, (1) sub-genus *Eu-Helminthosporium*, (2) sub-genus *Cylindro-Helminthosporium*. Although Nisikado did not examine all the species which we have worked upon, and hence any generalisation may be somewhat inaccurate, it is interesting to find that four of those seven species of *Helminthosporium* which we have found to give good yields of hydroxyanthraquinones, *i.e.*, *H. gramineum*, *H. catenarium*, *H. tritici-vulgaris* and *H. avenae*, are placed by Nisikado in the sub-genus *Cylindro-Helminthosporium*. The other three species, *H. cynodontis*, *H. euchlaenae* and *H. velutinum*, were not examined by Nisikado. On the other hand, twelve of the sixteen species giving entirely negative results, and four of the ten species which only give traces of hydroxyanthraquinones are placed by Nisikado in the sub-genus *Eu-Helminthosporium*. The remaining species were not examined by Nisikado with the exception of *H. teres* Sacc. which is placed by him in the

sub-genus *Cylindro-Helminthosporium*, and which we found to give only traces of hydroxyanthraquinones.

The molecular constitutions of helminthosporin (I) and of cynodontin (II) have already been satisfactorily elucidated [1933, 1, 2], but that of catenarin (III), formerly known as hydroxy*iso*helminthosporin, was left unsettled by previous work. Evidence is now presented, showing that catenarin must be a β-hydroxymethyl-1:4:5-trihydroxyanthraquinone having one of the formulae (III), (IV) or (V). The evidence for this is briefly as follows.



Distillation of catenarin with zinc dust yields β-methylanthracene in rather small quantities—relatively less than helminthosporin. As has been previously reported [Charles *et al.*, 1933], all efforts to oxidise the side-chain in catenarin acetate to carboxyl failed; in every case most of the starting material was recovered unchanged, the rest being completely destroyed. The oxidation of catenarin with concentrated nitric acid was also studied, but apart from a minute quantity of a brownish-yellow substance—apparently a tetranitrocatenarin—the only solid product which could be isolated was oxalic acid. The behaviour of catenarin towards such oxidising agents as nitric acid and chromic anhydride is strongly reminiscent of that of morindone [Simonsen, 1918], which has been shown by synthesis [Jacobson and Adams, 1925; Bhattacharya and Simonsen, 1927] to be 1:5:6-trihydroxy-2-methylanthraquinone. Similarly, the methyl group in cynodontin acetate resists oxidation [Raistrick *et al.*, 1933, 1]. It is therefore reasonable to assume that catenarin contains a nuclear hydroxyl adjacent to a β-methyl or β-hydroxymethyl group. Further evidence for the structure of catenarin may be stated briefly as follows:

(1) Catenarin is unaffected by heating for 8 hours at 100° with methyl iodide and sodium methoxide. Thus, despite its solubility in aqueous sodium carbonate, catenarin appears to contain no β-hydroxyl groups, as these are methylated under such conditions.

(2) Some support for the view that catenarin contains a hydroxymethyl group is afforded by the fact that its acetate, on heating with a saturated solution of hydrogen bromide in acetic acid, yields a product containing bromine. As this product is not homogeneous and the bromine content is low, this evidence is inconclusive.

(3) On oxidation with manganese dioxide and concentrated sulphuric acid, catenarin gives a hydroxycatenarin (VI), $C_{15}H_{10}O_7$, which is but a feeble mordant

dyestuff. Any anthraquinone derivative containing five nuclear hydroxyls is of necessity a derivative of alizarin and it follows that hydroxycatenarin can contain only four, and catenarin, consequently, only three, nuclear hydroxyl groups. The colour of its solution in concentrated sulphuric acid—blue, with a red fluorescence—taken in conjunction with its feeble dyeing properties, indicate that hydroxycatenarin is probably a derivative of 1:4:5:8-tetrahydroxy-anthraquinone. Its production from catenarin in the manner described limits the possible formulae for the latter substance to the three expressions III, IV and V.

(4) That catenarin, like helminthosporin and cynodontin, contains nuclear hydroxyls in the 1 : 4 : 5-positions, is further borne out by the similarity of the absorption curves of the three substances in concentrated sulphuric acid solution (see Fig. 1).

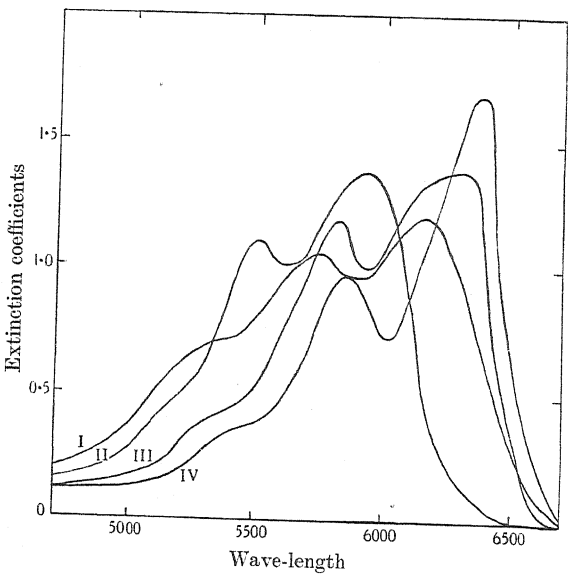


Fig. 1. I. Catenarin. II. Helminthosporin. III. Cynodontin. IV. Hydroxycatenarin.

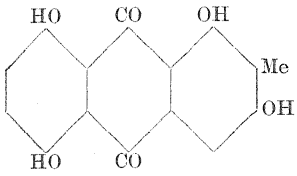
Constitution of tritisporin. This substance, of which only a small amount has been available for investigation, appears to have the formula $C_{15}H_{10}O_7$; it has been analysed as its pyridine salt and as a penta-acetyl derivative. It is only a weak adjective dyestuff and cannot be an alizarin derivative. Furthermore, although readily soluble in aqueous sodium carbonate, its acetate is insoluble in alkaline solutions. Hence the substance does not contain a carboxyl group. On distillation with zinc dust it affords β-methylanthracene.

The colour reactions and fluorescent properties are generally similar to those of catenarin and we are of the opinion that tritisporin must be a β-(hydroxy-methyl)-derivative of 1:3:5:7-, 1:3:6:8-, 1:4:5:8- or 1:3:5:8-tetrahydroxy-anthraquinone. The 1:4:5:8-orientation is at once excluded because this arrangement is required for hydroxycatenarin (VI), which is isomeric with, but very different from, tritisporin.

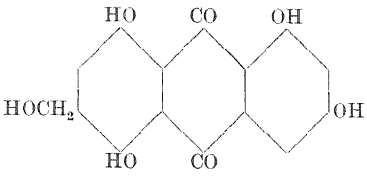
The colour reactions of tritisporin are also very divergent from those of anthrachrysone as described in the literature. For further confirmation we prepared a substance which was undoubtedly 1:3:5:7-tetrahydroxy-2:6-

dimethylanthraquinone (dimethylanthrachrysone) by the action of sulphuric acid at 100° on 2:6-dihydroxy-*p*-toluic acid. The product was sublimed in orange needles and gave a yellow non-fluorescent solution in acetic acid, an orange-yellow solution in aqueous sodium carbonate, a reddish orange solution in aqueous sodium hydroxide and a deep cherry-red solution in sulphuric acid. This behaviour is very unlike that of tritisporin, and it is improbable that the latter can be a derivative of anthrachrysone. It seems that 1:3:6:8-tetrahydroxyanthraquinone is unknown, but it would probably resemble anthrachrysone, much as chrysazin resembles anthrarufin.

For these reasons we consider that tritisporin is a derivative of 1:3:5:8-tetrahydroxyanthraquinone. It happens that a pure, analysed specimen of 1:3:5:8-tetrahydroxy-2-methylanthraquinone (VII) was available (unpublished synthesis by Dr E. H. Charlesworth) and this was found to be generally



VII



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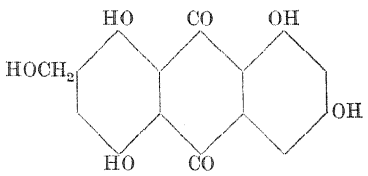

IX

similar and yet somewhat different from tritisporin in its reactions. In fact the substance VII resembled catenarin more closely in respect of the colour of its solution in sulphuric acid.

All the pigments isolated from species of *Helminthosporium* give fluorescent solutions in acetic acid, a property dependent on the quinizarin grouping. The differences existing between catenarin and VII on the one hand and tritisporin on the other suggest that catenarin should be represented by the formula III and one of the constitutions VIII or IX should be attributed to tritisporin.

Experimental.

A quantity of Czapek-Dox medium of the following composition was made up: glucose, 50·0 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4, 7H_2O$, 0·5 g.; $FeSO_4, 7H_2O$, 0·01 g.; distilled water, 1000 ml. 350 ml. quantities of this medium were distributed in each of a number of 1-litre conical flasks and sterilised. A measured quantity, previously determined, of sterile *N* sodium hydroxide solution was added to each flask to bring the p_H of the medium to 8·0. Five flasks were then each sown with a suspension in sterile Czapek-Dox medium of a pure culture of the species of *Helminthosporium* under investigation. The inoculated flasks were incubated at 24° for about 7 weeks, filtered, the mycelium dried in a vacuum-oven, ground to a fine powder and exhaustively extracted with chloroform in a Soxhlet apparatus. The chloroform extract was evaporated to dryness and portions of the dry extract were tested with strong sulphuric acid, aqueous sodium hydroxide solution, and concentrated ammonia. As mentioned in the introduction, a large number of species gave either entirely negative colour reactions, or where positive gave such small yields of hydroxyanthraquinones as to render isolation impracticable. In these cases no further investigation was carried out. In a few cases however considerable yields of

hydroxyanthraquinones were produced and these have been more exhaustively studied. The species chosen for further investigation were:

(*a*) *H. gramineum* Rabenhorst.
(*b*) *H. cynodontis* Marignoni.
(*c*) *H. euchlaenae* Zimmermann.
(*d*) *H. catenarium* Drechsler.
(*e*) *H. tritici-vulgaris* Nisikado.
(*f*) *H. velutinum* Link.
(*g*) *H. avenae* Eidam.

The results obtained with species (*a*), (*b*) and (*c*) have already been reported, and those obtained with (*d*), (*e*), (*f*) and (*g*) form the subject of the present communication.

In each case the cultural conditions, methods of separation and isolation of the crude pigment were similar and were as follows: 100 one-litre conical flasks, each containing 350 ml. Czapek-Dox medium, adjusted to p_H 8·0, were sown with the species under investigation and incubated at 24°. At the end of the incubation period the mycelium was filtered off, washed, pressed, dried in a vacuum-oven and ground to a fine powder in a coffee mill. The powdered mycelium was extracted in a large metal Soxhlet apparatus with chloroform until the chloroform extracts were colourless. The extraction usually occupied several days, and a series of solid fractions separating from the chloroform was obtained. These fractions were then examined for their hydroxyanthraquinone content. The final chloroform mother-liquors were evaporated to dryness and treated with light petroleum, by which means a considerable quantity of "fat" was isolated. The experimental details for each of the species examined are as follows.

(A) *Helminthosporium catenarium* Drechsler.

The culture used was purchased from Baarn and had been received by them in 1929 from Nisikado, who isolated it from *Beckmannia*. Incubation period, 65 days. Residual glucose, 0·65 %. The surface of the mycelium was flame-coloured and slightly buckled with a chocolate-brown reverse. Weight of mycelium from 97 flasks, 204 g. The chloroform extract was bright orange in colour and gave the following fractions:

(*a*) Fraction 1. First day's extraction. Weight, 9·96 g.; M.P. 220–226°.
(*b*) Fraction 2. Second and third day's extraction. Weight, 3·72 g.; M.P. 244–245°.
(*c*) Fraction 3. Fourth, fifth and sixth day's extraction. Weight, 6·10 g.; M.P. 193–205°.
(*d*) Fraction 4. *Ex.* mother-liquors from fractions 1, 2 and 3. Weight, 2·35 g.; M.P. 193–198°.

The extraction in the Soxhlet proved very tedious, and hence the mycelium was now re-extracted by boiling with a large volume of chloroform giving:

(*e*) Fraction 5. Weight, 11·29 g.; M.P. 244–247°, and
(*f*) Fraction 6. Weight, 9·30 g.; M.P. 244–246°.

Even now the pigment was not completely extracted, and hence the incompletely extracted mycelium was dried and re-extracted with a large volume of boiling acetone and there were thus obtained:

(*g*) Fraction 7. Weight, 4·72 g.; M.P. 238–241°, and
(*h*) Fraction 8. Weight, 1·55 g.; M.P. 179–181°.

Total weight of crude pigment, 48·99 g.

There were also obtained from the light petroleum extract 16·10 g. of crude "fat."

The colours developed by the various fractions on dissolution in conc. sulphuric acid indicated the probable presence in the crude pigmented material of hydroxy*iso*helminthosporin—henceforth called catenarin, as it is the characteristic product of *H. catenarium*—and helminthosporin. On this basis fractions 1 and 2 consist of catenarin contaminated with a small quantity of helminthosporin; fraction 3 is similar, but contains rather more helminthosporin, whilst fractions 4 and 8 are almost entirely composed of this pigment. Fractions 5 and 6

consist of nearly pure catenarin, and fraction 7 is somewhat similar but contains a trace of helminthosporin. For the isolation of the two pigments in a pure state, fractions 4 and 5 were employed.

Catenarin. The crude material was recrystallised from alcohol, when it formed red, slender plates containing alcohol of crystallisation which it lost slowly in air. It had M.P. 246° and agreed in all its properties with "hydroxy*iso*helminthosporin" (M.P. 246°) from *H. gramineum*; a mixed M.P. with the latter substance showed no depression. (Found in material dried at 110°: C, 63·1; H, 3·7 %. $C_{15}H_{10}O_6$ requires: C, 62·9; H, 3·5 %.)

Catenarin acetate. The crude pigment was acetylated in the usual way with acetic anhydride containing a trace of concentrated sulphuric acid. After recrystallisation from glacial acetic acid, the acetate was obtained as small lemon-yellow rods, M.P. 234–235° alone or mixed with "hydroxy*iso*helminthosporin" acetate (M.P. 234–235°). (Found: C, 61·1; H, 4·1 %. $C_{23}H_{18}O_{10}$ requires: C, 60·8; H, 4·0 %.)

Isolation of helminthosporin. Helminthosporin acetate. The crude pigment, on acetylation with acetic anhydride containing a trace of concentrated sulphuric acid, yielded an acetate, which, after three recrystallisations from glacial acetic acid, formed a mass of fluffy yellow needles, M.P. 225°. (Found: C, 63·6; H, 4·1 %. $C_{21}H_{16}O_8$ requires: C, 63·6; H, 4·0 %.) It had all the properties of helminthosporin acetate, and the mixed M.P. with this substance showed no depression.

Helminthosporin. The above acetate was hydrolysed by warming on the water-bath with 15 % sodium hydroxide during 90 minutes. The deep violet solution was acidified with hydrochloric acid and, after boiling for a few minutes to ensure coagulation, the red-brown precipitate was collected, washed with water, dried and twice recrystallised from pyridine. The product formed flat, maroon-coloured needles with a bronze lustre, M.P. 226–227°. (Found: C, 66·8; H, 3·8 %. $C_{15}H_{10}O_5$ requires: C, 66·7; H, 3·7 %.) It had all the properties of helminthosporin (M.P. 226–227°), and showed no alteration in M.P. when mixed with a sample of the latter substance.

(B) *Helminthosporium tritici-vulgaris* Nisikado.

The culture used was purchased from Baarn and was received by them in November 1929 from Nisikado, strain number 285, who isolated it from diseased wheat, *Triticum vulgare*. Incubation period, 39 days. Residual glucose, 0·88 %. The surface of the mycelium was buff-coloured, somewhat crumpled and with a chocolate-coloured reverse. Weight of mycelium from 100 flasks, 204 g.

The chloroform extract gave the following fractions:

(*a*) Fraction I. Separating from the combined chloroform extracts after 6 days' extraction. Weight, 9·04 g.; M.P. 216–225°.

(*b*) Fraction II. Separating from mother-liquors from fraction I after evaporation to low bulk. Weight, 0·30 g.; M.P. 152–176°.

(*c*) Fraction III. The mother-liquors from fraction II were evaporated to dryness and extracted with light petroleum giving an insoluble residue, fraction III, weight 1·85 g.; M.P. 128–140°.

Total weight of crude pigment, 11·19 g.

There were also obtained from the light petroleum extract 39·3 g. of crude "fat."

Preliminary examination of the colours produced on dissolving samples of the three fractions in concentrated sulphuric acid indicated that fraction I probably consisted largely of catenarin, but the reddish tinge of the solution suggested that some other substance was also present; fractions II and III gave red-violet solutions with weak red fluorescence and appeared to contain some helminthosporin.

The method of isolation of the pigments was the following:

Fraction I was extracted with alcohol in a Soxhlet apparatus for about 3 hours, after which time the liquid siphoning over became distinctly lighter in colour, and the extract was allowed to crystallise. A brownish material (A) was left as unextracted residue. The reddish-brown crystals separating from the alcohol extract were ground and again extracted with alcohol in a Soxhlet, the process being interrupted when a small amount (*ca.* 0·5 g.) remained in the thimble; this residue was combined with (A). The alcohol extract deposited on standing brownish-red plates (B).

Isolation of tritisporin from (*A*). The crude material had an indefinite M.P. 250–260° and contained appreciable quantities of a greyish impurity which was not readily removed by simple recrystallisation. Accordingly the crude substance was acetylated in the usual way with acetic anhydride containing a trace of sulphuric acid, and after several recrystallisations from glacial acetic acid *tritisporin acetate* was obtained as a mass of very fine pale yellow needles, M.P. 215°. (Found: C, 58·5; H, 3·8 %. $C_{25}H_{20}O_{12}$ or $C_{15}H_5O_2(OAc)_5$ requires: C, 58·6; H, 3·9 %.) The substance was insoluble in cold dilute sodium carbonate solution.

This acetate was hydrolysed by heating on the water-bath with aqueous sodium hydroxide (15 %) during 90 minutes. The blue-violet solution was then acidified and boiled for a few minutes to coagulate the reddish-brown precipitate which was collected, washed with water and dried. The product was sparingly soluble in acetic acid and alcohol but the best solvent for recrystallisation was pyridine from which the *tritisporin* separated as long narrow reddish-brown plates with a bronze lustre, apparently as a pyridine salt. (Found: C, 63·6; H, 4·0; N, 3·8 %. $C_{15}H_{10}O_7.C_5H_5N$ requires: C, 63·0; H, 3·9; N, 3·7 %.) Tritisporin separates from alcohol or acetic acid in flat needles slightly browner in colour than the pyridine salt; it has M.P. 260–262° but the M.P. is difficult to determine accurately as considerable sublimation occurs from 250° onwards.

Tritisporin dissolves very readily in cold sodium carbonate solution to a violet-red solution and in caustic soda to a blue-violet solution which is slowly decolorised in the air. Its solution in concentrated sulphuric acid is reddish violet, redder in tone than catenarin solutions, and on addition of boric acid it becomes blue in thin layers and violet in thick layers. Solutions of tritisporin in acetic acid are salmon-pink with a yellowish green fluorescence.

2-Methylanthracene from tritisporin. Tritisporin (120 mg.) was subjected in portions of 20 mg. to distillation with electrolytic zinc dust according to the micro-zinc dust distillation method of Kögl [1924]. The product of each experiment was deposited in the cooler parts of the tube as a mass of yellowish leaflets with a greenish fluorescence. The combined distillates were extracted from the tubes with ether, the solution filtered and the ether removed on the water-bath. The crystalline residue (*ca.* 10 mg.) was now recrystallised from alcohol. After three crystallisations it was obtained as glittering almost colourless leaflets, M.P. 200–201°, which showed all the properties of 2-methylanthracene. The mixed M.P. with a specimen of synthetic 2-methylanthracene (M.P. 203–204°) was 203° while a mixture with anthracene melted at 188–190°.

Isolation of catenarin from (*B*). The crude brownish red material was acetylated in the normal way by heating with acetic anhydride containing a trace of sulphuric acid. After three recrystallisations from acetic acid the acetate was obtained as small lemon-yellow rods, M.P. 234–235° alone or mixed with catenarin acetate.

The above acetate was hydrolysed by heating with aqueous sodium hydroxide (15 %) on the steam-bath during 90 minutes. The deep violet solution was acidified, boiled for a few minutes and the red-brown precipitate collected, washed with water, dried and recrystallised from alcohol. The recrystallised product formed slender red plates containing alcohol of crystallisation and had M.P. 246°. (Found in material dried at 110°: C, 63·0; H, 3·2 %. $C_{15}H_{10}O_6$ requires: C, 62·9; H, 3·5 %.) It had all the properties of catenarin and the mixed M.P. with a specimen of the latter substance showed no depression.

Preparation of helminthosporin acetate from fractions II and III. Fractions II and III showed a weak red fluorescence when dissolved in concentrated sulphuric acid, and the presence of helminthosporin was suspected. As the quantity present appeared to be small, however, the direct isolation of helminthosporin by crystallisation was deemed impracticable. Accordingly the combined material of the two fractions was extracted with alcohol for a short time to remove any readily soluble impurities and then acetylated in the usual way with acetic anhydride containing sulphuric acid. The yellow acetate was now separated into two portions by fractional crystallisation from acetic acid. The less soluble part, which consisted mainly of catenarin acetate, was discarded. The more soluble fraction which contained the helminthosporin acetate was subjected to repeated crystallisation from acetic acid, and a small amount was finally obtained in pale yellow fluffy needles, M.P. 224–225°, which had all the properties of helminthosporin acetate (M.P. 225°). The mixed M.P. with a sample of helminthosporin acetate was unchanged (a mixture with catenarin acetate melted at *ca.* 200°).

The presence of helminthosporin in the products from *H. tritici-vulgaris* is thus demonstrated.

Owing to unavoidable losses entailed in the separation of the three pigments an accurate analysis cannot be submitted; the coloured material from *H. tritici-vulgaris* is however estimated to consist of 75–80 % catenarin, 20–25 % tritisporin and 2–4 % helminthosporin.

Comparison of colours and fluorescences of solutions of various natural and synthetic pigments of the anthraquinone group in acetic acid.

Quinizarin	Orange-yellow; green fluorescence
Alizarin	Orange-yellow; no fluorescence
Anthrarufin	Yellow; no fluorescence
Chrysazin	Yellow; no fluorescence
1-Hydroxyanthraquinone	Pale yellow; no fluorescence
Hydroxyanthrarufin	Pure yellow; no fluorescence
Purpurin	Deep orange-yellow; weak green fluorescence
Emodin	Greenish yellow; no fluorescence
Morindone	Yellow; no fluorescence
2-Methyl-1:3:5:8-tetrahydroxyanthraquinone	Orange-yellow; strong green fluorescence
Helminthosporin, Catenarin	Brighter orange-yellow, red shade in thin layers; green fluorescence
Tritisporin	Salmon-red; yellowish-green fluorescence
Cynodontin, Hydroxycatenarin	Bluish red, crimson in thin layers; weak greenish yellow fluorescence

It will be observed that fluorescence is characteristic of quinizarin derivatives.

(C) *Helminthosporium velutinum* Link.

The culture used was purchased from Baarn, where it was isolated in July 1929 from *Salix* spec. Incubation period, 83 days. Residual glucose, 0·77 %. This species gave a thick, glutinous orange-red mycelium with an orange-red reverse. Weight of mycelium from 95 flasks, 297 g.

The chloroform extract had an orange-red colour indistinguishable from that given by *H. catenarium*, and gave the following fractions:

(*a*) Fraction 1. Separating from the combined chloroform extracts after 6 days' extraction. Weight, 11·88 g.; M.P. 244–245°.

(*b*) Fraction 2. Separating from mother-liquors from fraction 1 after evaporation to low bulk. Weight, 0·67 g.; M.P. 115–140°.

(*c*) Fraction 3. The mother-liquors from fraction 2 were evaporated to dryness and extracted with light petroleum giving an insoluble residue, fraction 3, weight 2·13 g.; M.P. 126–130°.

Total weight of crude pigment, 14·68 g.

There were also obtained from the light petroleum extract 64·9 g. of crude "fat."

Fractions 1 and 2 of the crude material dissolved in concentrated sulphuric acid to give solutions with the characteristic blue-purple colour shown by catenarin under these conditions. Fraction 3 was lighter in colour than the others, and its sulphuric acid solution was slightly redder and had a weak greenish fluorescence. Fraction 1 furnished, on recrystallisation from alcohol, catenarin, whose identity was established by analysis, M.P. and mixed M.P., and by preparation of its acetate. Fraction 3 contained a colourless substance, which was isolated and shown to be ergosterol (M.P., mixed M.P., colour reactions, and formation of acetate). The remainder of fraction 3, after removal of the ergosterol, consisted essentially of catenarin.

Catenarin. Fraction 1 crystallised from alcohol in red, slender plates containing alcohol of crystallisation, which was lost slowly in air. It had M.P. 246°, alone or mixed with catenarin (M.P. 246°; from *H. catenarium*), and showed all the properties of the latter. (Found in material dried at 110°: C, 63·0; H, 3·8 %. $C_{15}H_{10}O_6$ requires: C, 62·9; H, 3·5 %.)

The above material, on acetylation with acetic anhydride containing a trace of concentrated sulphuric acid, yielded an acetate which crystallised from glacial acetic acid in small, lemon-yellow rods, M.P. 234–235°. The M.P. was not depressed by admixture with catenarin acetate.

Examination of fraction 3. The material (2 g.) was extracted in a Soxhlet with light petroleum (B.P. 40–60°) during several hours.

(A) *Residue.* The residue in the thimble was red in colour, and on heating softened about 200° and melted finally *ca.* 230°. On acetylation, followed by repeated recrystallisation of the product from acetic acid, it gave catenarin acetate, M.P. 234–235°.

(B) *Extract.* The light orange-red light petroleum extract deposited, on cooling, a quantity of faintly pink crystals (0·5 g.). After several recrystallisations from alcohol this product was obtained as colourless, glittering leaflets, M.P. 161–162°. The mixed M.P. with an authentic specimen of ergosterol (M.P. 160–161°) was 161°, and a comparison of the colour reactions (Salkowski, Tortelli-Jaffé, Liebermann-Burchard, Rosenheim) of the two specimens revealed no divergences.

For further proof that the above product was indeed ergosterol, a portion of it (0·1 g.) was acetylated by boiling for one hour with acetic anhydride (0·5 ml.). On cooling, the acetate separated in glistening plates and, after recrystallisation from alcohol, had M.P. 178–179°.

The peculiar colour of the crude fraction 3 in sulphuric acid is explained by the presence of ergosterol. The colour could be artificially produced by adding ergosterol to solutions of catenarin in sulphuric acid.

(D) *Helminthosporium avenae* Eidam.

The culture used was isolated in 1925 by Mr F. T. Brooks, School of Botany, Cambridge, from oats (*Avena sativa* L.) showing signs of leaf blight disease. As the yield of pigment given by this organism was very small, three separate experiments using a total of 252 flasks were carried out. The average incubation period was 12 weeks. The residual glucose varied from 0·05 to 0·40 %. The mycelium of this species was greyish-white on the surface with a red-black reverse. The total weight of mycelium from 252 flasks was 941 g. The pigment was readily extracted with chloroform, and the chloroform extracts, which had a brilliant eosin-red colour reminiscent of cynodontin, were evaporated to moderate bulk and from this there separated:

(*a*) Fraction 1. Weight, 3·60 g.; M.P. about 250°.

(*b*) Fraction 2. The mother-liquors from fraction 1 were evaporated to low bulk, giving fraction 2, weight 1·92 g.; M.P. 245–260°.

(*c*) Fraction 3. The mother-liquors from fraction 2 were evaporated to dryness and extracted with cold light petroleum. The insoluble portion, representing fraction 3, weighed 0·66 g.; M.P. 135–145°.

Total weight of crude pigment from 252 flasks, 6·18 g.

There was also obtained from the light petroleum solution a total weight of crude "fat" of 165·9 g.

Fractions 1 and 2, from their appearance, and the colour of their sulphuric acid solutions—pure blue with a red fluorescence—appeared to consist almost entirely of cynodontin or of hydroxycatenarin. The presence of the latter was however ruled out by the insolubility of the material in cold, aqueous sodium carbonate. Recrystallisation of the crude pigment from pyridine yielded cynodontin, identified by M.P., mixed M.P., and by preparation of its acetate. In order to verify that the colourless fraction 3 was indeed ergosterol, it was further purified and compared with an authentic specimen of the latter compound; its acetate was also prepared.

Cynodontin. The crude material was recrystallised several times from pyridine, when it formed brown leaflets with a fine bronze lustre, M.P. 260°. (Found: C, 62·9; H, 3·4 %. $C_{15}H_{10}O_6$ requires: C, 62·9; H, 3·5 %.) This product had all the properties of cynodontin, and its M.P. was not depressed on mixing with the latter substance.

The above material, on acetylation with acetic anhydride containing a trace of concentrated sulphuric acid and recrystallisation from glacial acetic acid, gave an acetate forming bright yellow needles or rods, M.P. 224–225° alone or mixed with cynodontin acetate (M.P. 224–225°).

Fraction 3. Ergosterol. Recrystallisation of this, first from light petroleum, then from alcohol, gave colourless, glittering leaflets, M.P. 161–162°, showing no divergence in reactions from a specimen of ergosterol (mixed M.P., colour reactions of Salkowski, Tortelli-Jaffé, Liebermann-Burchard, Rosenheim). On boiling with acetic anhydride for one hour, it gave an acetate crystallising from alcohol in colourless, glistening plates, M.P. 178–179°.

Further experiments with hydroxyisohelminthosporin (catenarin).

It has been found that the M.P. of hydroxy*iso*helminthosporin, previously given as 238° [Charles *et al.*, 1933], can be raised to 246° by conversion into its acetate, followed by hydrolysis with 15 % sodium hydroxide and recrystallisation from alcohol. (Found in material dried at 110°: C, 63·2; H, 3·3 %. $C_{15}H_{10}O_6$ requires: C, 62·9; H, 3·5 %.) As this substance only occurs in very small amount in the products from *H. gramineum*, but is the main coloured product of *H. catenarium*, it has been re-named *catenarin*.

2-Methylanthracene from catenarin. Catenarin (0·5 g.) was distilled with zinc dust at a dull red heat in a current of hydrogen. 2-Methylanthracene was deposited in the cooler part of the tube as colourless leaflets with a green fluorescence; the yield of product was low (total weight from three such experiments, 50 mg.). After recrystallisation from alcohol the substance had M.P. 200° and had all the properties recorded for 2-methylanthracene; a mixture with the latter compound (M.P. 203–204°) had M.P. 202–203°.

Oxidation of catenarin with manganese dioxide and sulphuric acid. Preparation of hydroxycatenarin. Catenarin (2 g.) was dissolved in concentrated sulphuric acid (15 ml.) and finely powdered manganese dioxide (1·5 g.) added gradually with constant stirring, the temperature being maintained at 60–70°. After all had been added, the mixture was kept at 60–70° during a further 15 minutes and then poured into water. A brownish violet precipitate formed, which, on boiling for a short time with water, became dark brown by hydrolysis. The crude product was recrystallised from pyridine and was thus obtained as dark red-brown crystals. For further purification this material was converted into its acetate by boiling for a few minutes with acetic anhydride containing a trace of sulphuric acid. After four recrystallisations from glacial acetic acid hydroxycatenarin acetate was obtained as small greenish-yellow rods, M.P. 233–234° (mixed M.P. with catenarin acetate, *ca.* 210–213°). (Found: C, 58·6; H, 4·0 %. $C_{25}H_{20}O_{12}$ requires: C, 58·6; H, 3·9 %.) Yield, 0·55 g. The above acetate (0·4 g.) was warmed on the water-bath for 90 minutes with 15 % sodium hydroxide solution (30 ml.), when it dissolved slowly to a deep blue solution. After acidifying with hydrochloric acid and boiling for a few minutes to coagulate the dark brown precipitate, the latter was collected and twice recrystallised from pyridine. It separated as small brownish leaflets with a bronze lustre; on heating it underwent partial sublimation above 280° and melted at about 320°. (Found in material dried at 110° in a high vacuum over P_2O_5: C, 59·2; H, 3·5 %. $C_{15}H_{10}O_7$ requires: C, 59·6; H, 3·3 %.)

The crystals separating from pyridine solutions of hydroxycatenarin contain pyridine which is only partly lost on drying at room temperature. In appearance and in many of its properties hydroxycatenarin resembles cynodontin. Thus, it dissolves in concentrated sulphuric acid to a pure blue solution with a red fluorescence, and it possesses very feeble dyeing properties—much feebler than catenarin, although it is admittedly less soluble than the latter substance. This renders it highly probable that hydroxycatenarin is a derivative of 1:4:5:8-tetrahydroxyanthraquinone, while its fifth hydroxyl group must be in the side-chain as it would otherwise be an alizarin derivative and consequently a powerful mordant dyestuff. In view of its properties hydroxycatenarin must be 1:4:5:8-tetrahydroxy-2-(hydroxymethyl)anthraquinone (VI).

Oxidation of catenarin with nitric acid. Catenarin (1·5 g.) was warmed on the water-bath with nitric acid (20 ml. sp. gr. 1·42). After the initial reaction had subsided the mixture was heated on a boiling water-bath till evolution of brown fumes had practically ceased, then evaporated to small bulk and poured into water, a slight brownish precipitate being formed. The mixture was filtered.

Recrystallisation of the solid from acetic acid afforded fine brownish yellow needles (7 mg.); on heating the substance charred at about 250° without melting. It may be a tetranitrocatenarin. (Found: N, 9·4 %. $C_{15}H_6O_{14}N_4$ requires: N, 9·9 %.)

The only solid product which could be isolated from the filtrate was oxalic acid.

Attempted methylation of catenarin with methyl iodide. Catenarin (0·4 g.) was heated at 100° for 8 hours in a sealed tube with methyl iodide (1 g.) and sodium methoxide (1 g. sodium dissolved in 10 ml. methyl alcohol). After cooling the contents of the tube were acidified and the red precipitate collected; it consisted entirely of unchanged catenarin.

Action of hydrobromic acid on catenarin acetate. A mixture of catenarin acetate (0·5 g.) and a saturated solution of hydrobromic acid in acetic acid (15 ml.) was heated in a sealed tube at 100° during 8 hours. The contents of the tube, which were red in colour owing to hydrolysis, were poured into water, and after boiling for a few minutes to coagulate the precipitate the latter was collected and recrystallised from alcohol. The product had an indefinite M.P. 215–220° and was clearly a mixture. (Found: Br, 6·3 %. $C_{15}H_9O_5Br$ requires: Br, 22·9 %.) It was found impossible to isolate a homogeneous bromo-compound from this product by crystallisation. In a second experiment an unsuccessful attempt was made to purify the crude reaction product by acetylation and fractional crystallisation of the acetate; no definite separation was effected in six recrystallisations.

Efforts to improve the method by varying the conditions met with no success.

Absorption spectra.

The absorption of catenarin, hydroxycatenarin, helminthosporin[1] and cynodontin in the visible region of the spectrum is shown in Fig. 1 (p. 562). The curves refer to solutions in concentrated sulphuric acid, the concentration in each case being approximately $0{\cdot}5 \times 10^{-4} M$. The close similarity of the curves in their essential features, indicates the close relationship existing between these compounds and may be regarded as strong support for the formulae advanced for catenarin and hydroxycatenarin. (The absorption spectra of 1-methyl-3:5:8-trihydroxyanthraquinone and the related 1:4:6-trihydroxyanthraquinone described by Dimroth and Fick [1916] differ widely from any of those here described.)

Summary.

1. Hydroxyanthraquinones have been found to be produced by various species of *Helminthosporium*, in addition to those previously examined, when grown in a culture medium containing glucose as the sole source of carbon. The mycelium of *Helminthosporium catenarium* Drechsler contains hydroxy*iso*-helminthosporin as the chief constituent of hydroxyanthraquinone nature. This substance is more appropriately named *catenarin* and its constitution as a β-(hydroxymethyl)-1:5:8-trihydroxyanthraquinone is supported by its oxidation to a hydroxycatenarin closely resembling cynodontin (1:4:5:8-tetrahydroxy-2-methylanthraquinone).

2. *Helminthosporium velutinum* Link also produces catenarin but in this case the mycelium was found to contain appreciable amounts of ergosterol.

[1] Graves and Adams [1923] condensed 3:6-dimethoxyphthalic acid and *m*-cresol to a dimethoxyhydroxy-2-methylanthraquinone which was demethylated to a substance, M.P. 227°, described as 1:5:8-trihydroxy-3-methylanthraquinone. The compound was obtained in small quantity and was not analysed. However, it should have been identical with helminthosporin, and through the courtesy of Prof. Adams, to whom we are greatly indebted, we have been able to compare the substance with natural and synthetic helminthosporin with the result that the three specimens have been found to be identical. Owing to the confusion caused by the different system of numbering, we overlooked this important work of Graves and Adams in our first communication on helminthosporin.

3. *Helminthosporium avenae* Eidam was found to produce cynodontin and ergosterol in small relative amount.

4. *Helminthosporium tritici-vulgaris* Nisikado yielded catenarin, a little helminthosporin and a new pentahydroxy-2-methylanthraquinone termed *tritisporin*. The constitution of this substance is discussed and it is regarded as a 1:3:5:8-tetrahydroxy-6- (or 7-) (hydroxymethyl)anthraquinone.

We desire to express our sincere thanks to Mr W. K. Anslow for much technical assistance during the preparation of the crude pigments.

REFERENCES.

Bhattacharya and Simonsen (1927). *J. Ind. Inst. Sci.* **10**, A, 6.
Charles, Raistrick, Robinson and Todd (1933). *Biochem. J.* **27**, 499.
Dimroth and Fick (1916). *Liebig's Ann.* **411**, 334.
Drechsler (1923). *J. Agric. Res.* **24**, 670.
Eidam (1891). *Der Landwirth.* **27**, 509.
Graves and Adams (1923). *J. Amer. Chem. Soc.* **45**, 2439.
Jacobson and Adams (1925). *J. Amer. Chem. Soc.* **47**, 283.
Kögl (1924). *Liebig's Ann.* **440**, 33.
Link (1809). *Mag. Ges. Naturf. Fr.* Berlin, **3**, 10.
Nisikado (1929, 1). *Ber. Chara Inst. landw. Forsch.* **4**, 103.
—— (1929, 2). *Ber. Chara Inst. landw. Forsch.* **4**, 111.
Raistrick, Robinson and Todd (1933, 1). *Biochem. J.* **27**, 1170.
—— —— —— (1933, 2). *J. Chem. Soc.* 488.
Simonsen (1918). *J. Chem. Soc.* **113**, 766.

LXXX. THE IRRADIATION OF A TURBOT VITAMIN A CONCENTRATE WITH HOMOGENEOUS ULTRA-VIOLET RADIATION.

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(*Received March 1st, 1934.*)

The fact that concentrates of vitamin A may contain a chromogenic substance which under special conditions can be made to give rise to an increase in the Carr-Price units of the concentrate has been reported from time to time especially during the earlier work on the irradiation of vitamin A. Hawk [1929] and Norris and Church [1930] reported that exposure to air and light had this effect. Drummond [1930] however was unable to obtain similar results. Heilbron *et al.* [1931] found that in liver oils, which with the antimony trichloride test showed a preponderance of the 572 $m\mu$ chromogen over that of the 606 $m\mu$, an increase in strength of the 606 $m\mu$ occurred after several months' storage together with a small increase in strength of the extinction coefficient of the oils at 328 $m\mu$. In the author's work it has been found possible with a turbot concentrate containing 60 % vitamin A to increase both the blue units and the absorption coefficient at 328 $m\mu$ above the data recorded for the purest vitamin A by Carr and Jewell [1933].

Experimental.

A Hilger E_3 all-quartz spectrometer with sector photometer and a condensed copper spark to supply the light source were used to determine the absorption spectra of the solutions used. An air-cooled quartz mercury vapour lamp was used as source of ultra-violet radiation. The lamp was used throughout in the "end on" position and placed as close as possible to the vitamin A solution which was contained in a quartz test-tube and kept stirred during the experiments by a magnetic stirrer; irradiation was thus accomplished in the complete absence of air. A sheet of "uvirol" glass transmitting within the range 300–390 $m\mu$ was interposed between the solution and the lamp and all solutions were irradiated with this radiation. The value of E is calculated from the relation $\log I_0/I = Ecd$ where C is expressed in mg./ml., d in cm.; that of the blue values is calculated for Lovibond blue units for 1 mg./ml. as in the paper of Woolf and Moore [1932]. All solutions were irradiated and their absorption spectra determined in spectroscopic alcohol. After irradiation the solutions were evaporated at 50° *in vacuo* and concentrated for E and blue value readings, the blue values being determined in chloroform in the usual manner, the reading being taken immediately after addition of the antimony trichloride reagent.

A turbot concentrate prepared by Dr T. Moore of Cambridge was used in these experiments. It was found that with solutions containing 0·0011 % vitamin A an exposure of 3 hours caused only a small decrease in E and blue units and that solutions containing 0·00011 % vitamin A, in the case of this

particular concentrate, were readily affected by short exposures; the latter concentration was therefore used throughout this investigation.

After 1 minute's irradiation both *E* and blue units have decreased, and the absorption band of the blue solution of the non-irradiated concentrate at 565 $m\mu$ has changed to a band at 570 $m\mu$. After 3 minutes' irradiation *E* and blue units have further decreased and the blue solutions show a band at 575 $m\mu$. On further irradiation both *E* and the blue units increase until a maximum is reached after 21 minutes' irradiation, the absorption band of the blue solutions being 575 $m\mu$ and the absorption band in the ultra-violet at 328 $m\mu$ slightly flattened out. After 28 minutes' irradiation the blue units and *E* have fallen, selective absorption having almost disappeared, the band of the blue solutions with antimony trichloride now reading 570 $m\mu$. Further irradiation caused general absorption and very low blue values.

During irradiation a product has thus been formed which as regards *E* and blue units does not differ from vitamin A. The behaviour of the concentrate is certainly evidence of its lack of homogeneity and that the substance absorbing at 565 $m\mu$, which was evidently in excess in the concentrate, is transformed into one absorbing at 575 $m\mu$; further the change involves both *E* and blue units in definite proportions, so that if the substance absorbing at 575 $m\mu$ is without biological activity, it might readily simulate vitamin A. It is also noteworthy that the concentrate was so readily influenced by dilution as regards its reaction under ultra-violet radiation.

REFERENCES.

Carr and Jewell (1933). *Nature*, **131**, 92.
Drummond (1930). *J. Soc. Chem. Ind.* **49**, 285 T.
Hawk (1929). *Science*, **59**, 200.
Heilbron, Gillam and Morton (1931). *Biochem. J.* **25**, 1352.
Norris and Church (1930). *J. Biol. Chem.* **89**, 421.
Woolf and Moore (1932). *Lancet* (ii), 13.

LXXXI. NOTE ON THE ALLEGED REVERSAL OF THE DENATURATION OF SERUM-ALBUMIN.

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(*Received March 1st, 1934.*)

THE claim to have effected the reversal of the denaturation of serum-albumin [Anson and Mirsky, 1931] is so contrary to general experience that it is worthy of close examination. The method consists in dissolving the denatured protein in dilute acid and neutralising with dilute sodium hydroxide solution at room temperature. A portion of the albumin treated in this way crystallises like untreated albumin and behaves similarly in the precipitin reaction [Miller, 1933]. At first sight this evidence appears convincing but it is necessary to consider the various steps in the process and the assumptions involved very critically, since the conclusions reached are of some importance in the study of proteins.

EXPERIMENTAL METHODS.

Serum-albumin.

The serum-albumin used was prepared from horse plasma. Globulins were removed by precipitation with 18 % of anhydrous sodium sulphate at 34°, and crystallisation of the albumin was effected by cautious addition of acetic acid to the filtrate in the ordinary way. Crystallisation of albumin is usually carried out in ammonium sulphate filtrates, but the sodium sulphate process is equally effective. The crystalline albumin was filtered off on cloths, lightly pressed and dialysed in cellophane bags. The resulting solution was brought to p_H 7 and filtered through a sterile Seitz filter. The resulting 800 ml. of sterile 10 % solution of albumin was used as a stock preparation in these and other experiments.

Electrometric p_H *determinations.*

A compact micro-quinhydrone electrode assembly was devised (Fig. 1). The cell is readily made by drawing out a piece of 7·5 mm. bore glass tubing and bending the ends round into the form of a U-tube. The bottom horizontal portion of the tube is filled with 3 % agar saturated with KCl and acts as a salt bridge. In one limb is placed a buffer solution of known p_H and in the other the solution to be investigated. About 0·3 ml. of solution is sufficient for a determination. A little quinhydrone is added to each limb and platinum wire electrodes of the usual form are placed in each limb, being supported by corks resting on the rims of the limbs. A Cambridge portable potentiometer completes the simple apparatus.

Effect of p_H *on denaturation.*

A neutral 0·4 % solution of serum-albumin in 0·8 % saline was titrated with 0·1 *N* HCl, and 4·5 ml. samples of different p_H values were withdrawn at various stages of the titration. For the p_H determinations the quinhydrone vessel described above was used. The samples were placed in 5 × 0·5 in. tubes in a rack immersed in a tank of boiling water from which the burner had just

been removed. The tubes were so arranged in the rack that samples of similar p_H were not adjacent. When the tubes had been in the hot water for 3 minutes they were transferred to a bath of cold water. The precipitates, when present, were filtered off and protein determinations on the filtrates were conducted by means of the phenol reagent of Folin and Ciocalteu. In Fig. 2 are plotted the

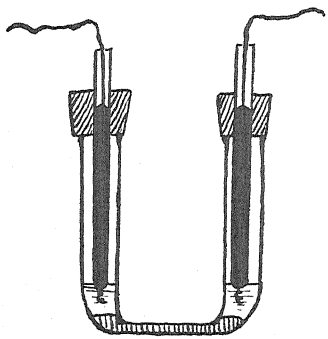

Fig. 1.

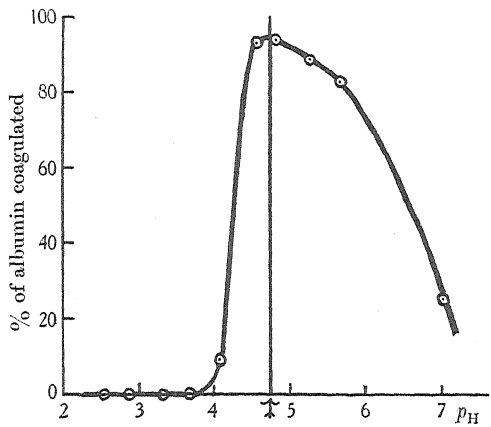

Fig. 2.

Fig. 1. Quinhydrone electrode vessel.

Fig. 2. Effect of p_H on heat-coagulation of albumin. (The vertical line, indicated by arrow, indicates the isoelectric point.)

amounts of precipitation at different levels of p_H. It will be observed that coagulation has been maximum in the region of the isoelectric point (p_H 4·5 to 5·0) and falls away slowly on the alkaline side but very steeply on the acid side. Thus, taking points equidistant from the isoelectric point (say p_H 4·75), at p_H 5·5 over 80 % of the protein is coagulated, whereas at p_H 4·0 only 9 % is precipitated. Heating at reactions more acid than p_H 4·0 does not effect coagulation when the solution is cooled and brought to the isoelectric point.

Effect of salts.

It is well known that coagulation of proteins on heating does not occur in the absence of salts and this is shown in the following experiment. The stock 10 % solution of albumin was diluted with distilled water giving a final concentration of 0·2 % albumin. Twelve samples were taken and their reactions adjusted with 0·02N HCl to p_H values ranging from 4·1 to 7·2. The samples were heated in a boiling water-bath for 15 minutes but no coagulation occurred. A small amount of salt was added to each of the cooled tubes without there being any coagulation, but when the tubes were reheated coagulation occurred, being almost complete in the samples of p_H from 4·6 to 5·0. Electrodialysed albumin is said to coagulate without added salt [Adolf, 1926]. The effect of salts on protein coagulation was not taken into account by Anson and Mirsky.

Modifications of Anson and Mirsky's experiment.

Unless the experimental conditions, especially p_H, are carefully selected it is not possible to repeat Anson and Mirsky's experiment, and some care is necessary in finding the appropriate conditions, since in their paper no p_H

measurements are recorded. The following experiment is of some interest and will be described in detail.

A 2 % solution of serum-albumin in 0·8 % saline was rendered acid (p_H 2·3) by addition of hydrochloric acid and heated for 4 minutes in a boiling water-bath. The solution whilst still hot was divided into three portions. One portion was brought to the isoelectric point by addition of sodium hydroxide to the hot solution and then immediately cooled (Part I), a second portion was brought to the isoelectric point and allowed to remain hot for 2 minutes before cooling (Part II), whilst the third portion was allowed to remain hot for 2 more minutes, then cooled and finally brought to the isoelectric point when cold (Part III).

Part I, which had been brought to the isoelectric point and immediately cooled was in the form of a granular precipitate; Part II which had been heated for 2 minutes at the isoelectric point was in the form of a gummy coagulum; whilst Part III, which had been heated in acid solution and cooled before being brought to the isoelectric point, was a perfectly clear solution. The method of reversal of denaturation described by Anson and Mirsky was applied to Parts I and II only, since Part III was already totally in solution. Part I dissolved readily in dilute hydrochloric acid giving a clear solution but Part II gave a highly viscous gel with which efforts at reversal were quite unsuccessful. When the acid solution of Part I was again brought to the isoelectric point by addition of dilute sodium hydroxide at room temperature a turbidity was produced, and, on addition of an equal volume of saturated ammonium sulphate, there was a considerable amorphous precipitation. Now when an equal volume of saturated ammonium sulphate solution was added to Part III the protein remained soluble and on cautious addition of dilute acetic acid in the usual way the albumin crystallised out in fine rosettes of needles, no amorphous material being detected microscopically. If the granular precipitate (Part I) be centrifuged down and ground by an agate pestle in an agate mortar some 40 % dissolves up in the supernatant fluid.

Comment on these experiments is made in the following section, but a point raised by Miller [1933] must be referred to here. He mentions the fact that, since the solubility of native albumin in half-saturated ammonium sulphate solution near the isoelectric point is limited, the precipitate obtained is not necessarily all denatured protein. As a matter of observation however the addition of an equal volume of saturated ammonium sulphate to serum-albumin at the isoelectric point (p_H 4·8) results in a considerable change in p_H (to p_H 5·2) and at this reaction undenatured albumin is soluble.

Using the disulphide reaction [Walker, 1925] and observations of optical rotatory power [Hewitt, 1927] evidence of reversal of denaturation was not obtained.

Discussion.

At the outset we are faced by the significant fact that, in order to observe the reversal of denaturation, it is necessary to adhere rigidly, not only to the conditions essential for the reversal, but also to Anson and Mirsky's methods of preparing the denatured albumin. The exact repetition of their experiments is rendered difficult by their neglect to record the p_H values of their solutions.

The first two methods employed by Anson and Mirsky for preparing denatured albumin, namely precipitation with trichloroacetic acid and with acidified acetone, need not be considered for the moment as there is no satisfactory evidence that the albumin has been denatured. The third method consists in warming the albumin in acid solution and adjusting the reaction of

the hot solution to the isoelectric point. If immediately cooled the precipitate may be redissolved in dilute acid preparatory to the reversal of the denaturation. This is effected by the addition of 0·1 *N* NaOH to the solution at room temperature until the isoelectric point is reached. It may seem that the partial neutralisation with dilute caustic soda at room temperature is not a process likely to effect a reversal of denaturation. The dissolving of the precipitate in dilute acid would seem more likely and, indeed, Northrop [1931] has reactivated a small portion (up to 1 %) of inactivated pepsin by treatment with acid. Actually however the crux of the matter has appeared at an earlier stage. The fundamental difficulty lies in the exact definition of a denatured protein. Anson and Mirsky's [1931] definition, "All denatured proteins are insoluble at their isoelectric points but soluble in acid or alkali," is not of general application, but it will serve our present purpose if accepted with suitable qualifications.

It is obvious that in order to prove the possibility of reversing the denaturation process it is essential to the argument to prove satisfactorily that the protein has in fact been denatured. Now Anson and Mirsky assume, without presenting any evidence to support the assumption, that when heated at 100° in acid solution for 4 minutes albumin is quantitatively denatured. It is a fact however that serum-albumin, after being treated in this way, then cooled down to room temperature and the reaction adjusted to the isoelectric point, gives no evidence of being denatured. The albumin is still soluble in water and dilute salt solutions at the isoelectric point, it is still soluble in 50 % ammonium sulphate and may be crystallised in the usual way. These facts would be explained by Anson and Mirsky as being due to the reversal of the denaturation during the neutralisation of the cold acid albumin solution. In order to avoid this reversal Anson and Mirsky neutralise the acid solution whilst it is still hot, and under these conditions protein is precipitated at the isoelectric point. They assume that reversal of denaturation is avoided by neutralising the hot instead of the cold solution. Reversal of the denaturation is now effected by dissolving the precipitate in dilute acid and then neutralising the cold solution. In this way some two-thirds of the original amount of protein may be recovered in the undenatured form. Anson and Mirsky's explanation however fails to explain why there should be quantitatively complete recovery of undenatured protein in the experiment mentioned above where the albumin heated in acid solution is cooled before neutralisation. Since the reversal process, namely the neutralisation of the cold acid solution, has been identical in each case the different yields of undenatured protein must be due to differences in the denaturation process. Anson and Mirsky suggest that the simplest explanation of their partial recovery of undenatured protein is that there are two effects of heating protein solutions, a reversible and an irreversible denaturation. There is however no necessity to introduce this complication. Let us consider for a moment a further modification of the experiment. If the hot albumin solution when brought to the isoelectric point, instead of being at once cooled, is kept hot for a minute or two longer, the character of the precipitate changes, and the denaturation cannot be reversed. (It should be emphasised that a longer period of heating the acid solution does not have this effect.) The simplest explanation of all the facts and observations is that the heating of the albumin in acid solution has little, if any, denaturing effect. Heating at the isoelectric point however results in very rapid denaturation, so that when the hot acid solution of albumin is brought to the isoelectric point, there is an immediate precipitation of granular floccules. These floccules consist of a shell or skin of denatured protein surrounding a core of undenatured albumin and when ground up in an agate mortar some 40 %

of the precipitate is dissolved. If the granular precipitate be immediately cooled, it dissolves readily in dilute acid, and when again neutralised the undenatured protein remains dissolved, whereas the denatured portion retains its characteristic properties, for example it is insoluble in half-saturated ammonium sulphate solution and is amorphous. If the experiment be varied by keeping the isoelectric solution hot for a minute or two before cooling, the denatured skin of the granular floccules can no longer protect the core and the protein in the interior of the floccules becomes denatured as well. This completion of denaturation is accompanied by a change in the appearance of the precipitate which now becomes more gummy. It is mentioned specifically by Anson and Mirsky that the portion of the protein (about one-third) which has not been reversed in the first instance, cannot be reversed by subsequent treatment. This portion corresponds to the denatured skin mentioned above and in the present experiments it was found that the longer the interval between the adjustment of the reaction to the isoelectric point and the cooling, the less protein could be recovered in the reversed condition. So far therefore there is no evidence that conflicts with the view that protein denaturation is an irreversible process and there is no evidence of two kinds of denaturation, reversible and irreversible.

At this point it is necessary to consider critically the evidence presented by Miller [1933] in support of the possibility of reversible denaturation. Using the precipitin reaction he concluded that native and "reversed" serum-albumin were similar but were different from denatured albumin. This putative evidence is however rendered void by the fact that the denatured protein he used was not a comparable control to the "reversed" albumin. The "reversed" albumin was prepared by Anson and Mirsky's method and the reversed portion had not therefore been denatured, whilst the "denatured" protein had been subjected to further heating processes at the isoelectric point, and was thus truly and irreversibly denatured.

It is clear therefore that the claim to have reversed the heat-denaturation of serum albumin has not been confirmed.

SUMMARY.

1. No evidence for the possibility of reversing the denaturation of serum-albumin was found.

2. Serum-albumin may be heated in acid solution without being appreciably denatured.

3. In experiments purporting to demonstrate partial reversal of denaturation evidence is lacking that the albumin recovered had been denatured.

The author is indebted to Dr R. G. White for his encouraging interest.

REFERENCES.

Adolf (1926). *Biochem. Z.* **170**, 126.
Anson and Mirsky (1931). *J. Gen. Physiol.* **14**, 725.
Hewitt (1927). *Biochem. J.* **21**, 216.
Miller (1933). *J. Exp. Med.* **58**, 625.
Northrop (1931). *J. Gen. Physiol.* **14**, 713.
Walker (1925). *Biochem. J.* **19**, 1082.

LXXXII. THE IDENTIFICATION OF AMINO-ACIDS BY MEANS OF 3:5-DINITROBENZOYL CHLORIDE.

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(*Received March 1st, 1934.*)

The present investigation arose as a result of an attempt to prepare an easily obtainable crystalline derivative of oxidised glutathione. The author [1933] has already shown that oxidised glutathione fails to give a dinaphthalenesulphonyl derivative with naphthalene-2-sulphonic chloride, the latter being reduced to naphthalene-2-sulphinic acid.

An attempt was made to prepare the *N*-dibenzoyl derivative of oxidised glutathione by shaking it with benzoyl chloride and alkali according to the usual Schotten-Baumann method. On acidifying the product, a sticky mass was obtained together with a copious evolution of hydrogen sulphide.

It was obvious that the peptide was being decomposed by the alkali, and hence it seemed advisable to cut down the time of benzoylation to a minimum, a condition which might be accomplished by using 3 : 5-dinitrobenzoyl chloride. Both on the Lapworth theory of alternating polarities [Lapworth and Shoesmith, 1922] and on the more modern electronic theories this should behave as a highly reactive acid chloride. Unfortunately no product could be isolated in the case of GSSG, but this method of benzoylation using 3 : 5-dinitrobenzoyl chloride was extended to many other amino-acids with success.

It was found to be an exceedingly rapid and efficient method, pale yellow or colourless crystalline derivatives, having sharp melting-points, being usually obtained. The method consists in dissolving the amino-acid in excess of alkali and adding the calculated quantity of finely powdered 3 : 5-dinitrobenzoyl chloride. The mixture is shaken vigorously for about two minutes. The dinitrobenzoyl chloride dissolves almost instantaneously and on acidification the 3 : 5-dinitrobenzoyl derivative separates. In most cases practically no 3 : 5-dinitrobenzoic acid is formed provided that the acid chloride is not in excess.

All the monoamino-monocarboxylic acids examined gave crystalline *N*-3 : 5-dinitrobenzoyl derivatives, which on hydrolysis with hydrochloric acid gave 3 : 5-dinitrobenzoic acid and the original amino-acid.

The behaviour of the monoamino-dicarboxylic acids was rather unexpected. In the case of aspartic acid no product was isolable, but a considerable quantity of 3 : 5-dinitrobenzoic acid was produced. A formaldehyde titration of the filtrate showed that much of the aspartic acid remained unchanged. Glutamic acid also reacted only partially with 3 : 5-dinitrobenzoyl chloride, but in this case a certain amount of the 3 : 5-dinitrobenzoyl derivative was actually isolated. As previously stated no product could be isolated from GSSG.

It seems from this that the NH_2 group in the monoamino-monocarboxylic acids is highly reactive since it reacts rapidly and more or less completely with

3 : 5-dinitrobenzoyl chloride in the presence of sodium hydroxide. The mono-amino-dicarboxylic acids (and some other "acidic" amino-acids such as tyrosine) appear to have a much less reactive NH_2 group since much (or all) of the 3 : 5-dinitrobenzoyl chloride is hydrolysed before any condensation can be effected.

This observation is substantiated by the fact that whereas aspartic acid gives no product, and glutamic acid reacts only partially, asparagine and glutamine (which are less acidic) react completely with one[1] molecule of 3 : 5-dinitrobenzoyl chloride giving high yields of the corresponding mono-dinitrobenzoyl derivatives, practically no 3 : 5-dinitrobenzoic acid being formed. Furthermore *meso*diamino-succinic acid gives a theoretical yield of the corresponding di-3 : 5-dinitrobenzoyl derivative.

The apparent lower reactivity of the NH_2 group in the "acidic" amino-acids compared with the reactive NH_2 group in the "neutral" amino-acids towards 3 : 5-dinitrobenzoyl chloride in alkaline solution may perhaps be due to the different state of ionisation of this group at the p_H employed. As the reaction is a very rapid one such differences might be detected, whereas in the slower ordinary benzoylations using benzoyl chloride, there is opportunity for an equilibrium to be disturbed.

Whatever the cause, this difference in behaviour has permitted of a smooth and complete separation of certain classes of amino-acids. For instance, glycine has been separated from a mixture of aspartic acid and glycine, the latter reacting preferentially with 3 : 5-dinitrobenzoyl chloride. The separation was not impaired by the presence of large quantities of sodium salts. It is advisable, but not necessary, to remove ammonium salts. They do not interfere with the reaction between 3 : 5-dinitrobenzoyl chloride and glycine, but some of the acid chloride may be wasted as 3 : 5-dinitrobenzamide. The latter, however, owing to its insolubility in sodium hydroxide does not contaminate the product.

It has not yet been definitely established whether there is any reaction between 3 : 5-dinitrobenzoyl chloride and gelatin in alkaline solution, but large quantities of the protein interfere with the reaction between the acid chloride and glycine. A weight of gelatin equal to the weight of glycine can however be present without any deleterious effect.

Glycine has also been separated from glutamic acid and asparagine from aspartic acid. Although glutamic acid does form a derivative, the glycine derivative is formed preferentially.

An efficient separation has also been effected between glycine and leucine, owing to the fact that 3 : 5-dinitrobenzoylleucine is precipitated from alkaline solution by dilute acetic acid, whereas 3 : 5-dinitrobenzoylglycine is not. Other possibilities in this direction become evident from details given in the experimental part.

Cystine reacted very rapidly with 3 : 5-dinitrobenzoyl chloride to give di-(3 : 5-dinitrobenzoyl)-cystine which was characterised by the formation of a gel in aqueous or alcoholic solutions.

One would expect a different type of derivative in the case of the thiolamino-acids. To determine the reaction of the SH group alone thiolacetic acid was used. A rapid reaction involving the SH group took place as shown by the disappearance of the nitroprusside reaction. On acidification an oil was obtained, which solidified on long standing and from its reactions appeared to be *S*-3 : 5-dinitrobenzoylthiolacetic acid. Wertheim [1929] was the first to use 3 : 5-dinitrobenzoyl chloride for the identification of simple mercaptans. His method however was

[1] There is no reaction between 3 : 5-dinitrobenzoyl chloride and the acid amide group as shown by absence of reaction with acetamide.

different from the above in that he used small quantities of pyridine as a catalyst instead of sodium hydroxide and found that it was necessary to fuse the reactants. It is obviously an advantage to use sodium hydroxide as the amino-acids are usually soluble in this medium. It should be pointed out that he did not use 3 : 5-dinitrobenzoyl chloride in conjunction with the amino group.

3 : 5-Dinitrobenzoyl chloride reacts with cysteine to produce what appears to be the *S*-derivative containing a free NH_2 group. Unlike the *N*-derivatives it does not possess a sharp melting-point. It is a light brown powder which is not easily recrystallised and cannot be recommended for identification purposes.

No reaction could be effected between tyrosine and 3 : 5-dinitrobenzoyl chloride. Neither phenol nor salicylic acid reacted with this reagent in alkaline solution.

Guanidine gives an almost theoretical yield of di-(3 : 5-dinitrobenzoyl)-guanidine. This derivative is insoluble in NaOH and so does not interfere with the identification of the amino-acids by this method.

The three aminobenzoic acids react almost instantaneously with 3 : 5-dinitrobenzoyl chloride, but homogeneous products are not obtained in the case of the *o*- and *p*-compounds. This problem will be discussed elsewhere.

The 3 : 5-dinitrobenzoyl derivatives cannot be analysed satisfactorily by the micro-Kjeldahl method, low results being obtained. Correct results were obtained by the micro-Dumas method.

Buehler *et al.* [1933] have recently described the 3 : 5-dinitrobenzoates of certain amines; these are salts and are distinct from the derivatives described above. The author was unable to obtain the 3 : 5-dinitrobenzoates of glycine and several other amino-acids, a result to be expected.

An attempt was made to prepare the 3 : 5-dinitrobenzoyl derivatives of the amino-acids using pyridine instead of NaOH as the condensing agent. On acidification in each case the same compound was obtained, which proved to be a very stable acid pyridine salt of 3 : 5-dinitrobenzoic acid ($2C_6H_3(NO_2)_2COOH.C_5H_5N$). This was confirmed by dissolving 3 : 5-dinitrobenzoic acid in excess of pyridine and acidifying, the same product being obtained. This compound had previously been prepared by Pfeiffer [1914].

3 : 5-Dinitrobenzoic acid and all the 3 : 5-dinitrobenzoyl derivatives here described gave an intense violet coloration with acetone and NaOH. This test could be used qualitatively to determine whether the 3 : 5-dinitrobenzoyl residue had entered the amino-acid molecule. The nature of this colour change is now being investigated.

Experimental.

3 : 5-Dinitrobenzoylglycine. 0·75 g. (1 mol.) of glycine was dissolved in 20 ml. *N* NaOH (2 mols.) and 2·32 g. (1 mol.) of finely powdered 3 : 5-dinitrobenzoyl chloride were added. The mixture was shaken vigorously in a stoppered bottle, whereupon the acid chloride dissolved almost immediately, the solution becoming deep red. The shaking was continued for about 2 minutes, the solution filtered if necessary and acidified with dilute hydrochloric acid. The dinitrobenzoyl derivative crystallised out immediately[1]. Yield 2·1 g. (80 % theoretical). It was recrystallised from water in long colourless silky needles: M.P. 179°. (Found: C, 37·6; H, 3·08; N, 14·66 %. $C_9H_7O_7N_3$, H_2O requires: C, 37·63; H, 3·13; N, 14·63 %. Dried at 100°. Found: H_2O 6·0 %. Calc. 6·27 %.)

[1] If an excess of 3 : 5-dinitrobenzoyl chloride were used, this appeared as 3 : 5-dinitrobenzoic acid in the precipitate produced on acidification and could be removed by shaking the precipitate in the cold with a mixture of five volumes of 50–60° light petroleum and two volumes of alcohol. The glycine derivative is insoluble in this mixture. If the acid chloride were not used in excess, it was usually found unnecessary to carry out this treatment.

Hydrolysis of 3 : 5-dinitrobenzoylglycine. The compound was boiled with HCl (2 vols. HCl, 1 vol. water) for 1¼ hours. On cooling 3 : 5-dinitrobenzoic acid crystallised out and was recrystallised from water: M.P. 207°. (Found: N, 13·1 %, $C_7H_4O_6N_2$ requires: N, 13·2 %.)

A formaldehyde titration of the filtrate showed that the hydrolysis to glycine was complete.

The 3 : 5-dinitrobenzoyl derivatives of the monoamino-monocarboxylic acids described below were prepared in a manner similar to the above. Each derivative dissolved in strong alkali to give a dark red colour discharged by acids. Dilute HCl was used for precipitation from the alkaline solution unless otherwise stated.

3 : 5-Dinitrobenzoyl-dl-*alanine.* Recrystallised from water in minute pale yellow needles: M.P. 177°. (Found: N, 14·88 %. $C_{10}H_9O_7N_3$ requires: N, 14·84 %.)

3 : 5-Dinitrobenzoyl-l-*leucine.* Precipitated by dilute acetic acid. Only slightly soluble in boiling water, from which however it can be recrystallised in long colourless needles. Recrystallised more readily from 50 % aqueous alcohol: M.P. 186–7°. (Found: C, 48·06; H, 4·6; N, 12·62 %. $C_{13}H_{15}O_7N_3$ requires: C, 48·0; H, 4·62; N, 12·92 %.)

3 : 5-Dinitrobenzoyl-d-*valine.* Precipitated by dilute acetic acid. Recrystallised from water in colourless feathery masses: M.P. 157–8°. (Found: N, 12·78 %. $C_{12}H_{13}O_7N_3$, H_2O requires: N, 12·76 %. Found: H_2O, 5·5 %. Calc. 5·47 %.)

3 : 5-Dinitrobenzoyl-l-*phenylalanine.* Precipitated by dilute acetic acid. In order to purify it, it was dissolved in alcohol, diluted with water until just cloudy and placed in the ice-chest to crystallise. Short colourless needles: M.P. 93°. (Found: N, 11·54; H_2O, 2·9 %. $C_{16}H_{13}O_7N_3$, 0·5 H_2O requires: N, 11·69; H_2O 2·45 %.)

3 : 5-Dinitrobenzoyl-l-*asparagine.* Asparagine reacts with one molecule of 3 : 5-dinitrobenzoyl-chloride to give the above compound using the method already described. Yield = 90 %. Recrystallised from water in very thin flat glistening leaflets: M.P. 196°. (Found: N, 17·19 %. $C_{11}H_{10}O_8N_4$ requires: N, 17·18 %.)

3 : 5-Dinitrobenzoyl-d-*glutamine.* Formed from one molecule of glutamine and one molecule of 3 : 5-dinitrobenzoyl chloride. Yield, 95 %. Recrystallised from water in colourless radiating needles: M.P. 217°. (Found: N, 16·38 %, $C_{12}H_{12}O_8N_4$ requires: N, 16·47 %.)

N-*3 : 5-Dinitrobenzoyl*-S-*benzylcysteine.* 1·05 g. of *S*-benzylcysteine were dissolved in 10 ml. of *N* NaOH and 1·15 g. of 3 : 5-dinitrobenzoyl chloride added. The mixture was shaken until all the acid chloride had dissolved. A strong mercaptanic odour was noticeable, owing presumably to the partial hydrolysis of the *S*-benzylcysteine by the alkali. The solution was filtered and acidified with dilute acetic acid, a white feathery crystalline precipitate being formed. Recrystallised from aqueous alcohol in long slender needles: M.P. 92°. It was subsequently found advantageous to carry out the reaction at 0°, in which case no mercaptanic odour was perceptible. The reaction was complete in a few minutes at this temperature. Yield, 90 %. (Found: N, 9·69; S, 7·26 %. $C_{17}H_{15}O_7N_3S$, $2H_2O$ requires: N, 9·52; S, 7·26 %.)

N-*3 : 5-Dinitrobenzoyl*-S-*ethylcysteine.* As for *S*-benzylcysteine. Precipitated by acetic acid. Recrystallised from aqueous alcohol in colourless needles: M.P. 65°. Hygroscopic. (Found: N, 12·26; S, 9·03 %. $C_{12}H_{13}O_7N_3S$ requires: N, 12·24; S, 9·33 %.)

3 : 5-Dinitrobenzoyl-l-*methionine.* Unlike *S*-ethyl- and *S*-benzyl-cysteine, methionine could be acylated at room temperature without any decomposition. Recrystallised from aqueous alcohol in slender silky needles: M.P. 94–5°; anhydrous substance has M.P. 150°. Very soluble in cold alcohol. (Found: C, 40·76; H, 4·08; N, 12·14; S, 8·98 %. $C_{12}H_{13}O_7N_3S$, 0·5 H_2O requires: C, 40·91; H, 3·98; N, 11·93; S, 9·09 %.)

3 : 5-Dinitrobenzoyl-l-*tryptophan.* 1 mol. of tryptophan reacted with 1 mol. of 3 : 5-dinitrobenzoyl chloride. An orange precipitate was produced on the addition of dilute acetic acid to the alkaline solution. Recrystallised from alcohol as an orange crystalline powder. Insoluble in boiling water. M.P. 233° (decomp.). (Found: C, 54·3; H, 3·54 %. $C_{18}H_{14}O_7N_4$ requires: C, 54·27; H, 3·52 %.)

Di-(3 : 5-dinitrobenzoyl) meso*diaminosuccinic acid.* 1 mol. of *meso*diaminosuccinic acid reacted with 2 mols. of dinitrobenzoyl chloride in the presence of 4 mols. of *N* NaOH. On the addition of dilute acetic acid to the alkaline solution a crystalline precipitate slowly formed. The filtrate gave no precipitate with HCl showing that no 3 : 5-dinitrobenzoic acid had been produced. The precipitate was insoluble in boiling water, alcohol, acetone, benzene and glacial acetic acid. It dissolved in alkali with the production of a deep red colour. In order to purify it, it was dissolved in NaOH,

water was added until the red colour was just discharged and dilute acetic acid was added to reprecipitate it as a colourless crystalline powder. Melted with decomp. 254°. (Found: N, 16·03 %. $C_{18}H_{12}O_{14}N_6$ requires: N, 15·67 %.)

Di-(3 : 5-dinitrobenzoyl)-d-arginine. 1 mol. of arginine hydrochloride reacted with 2 mols. of the acid chloride. Precipitated from the alkaline solution by dilute acetic acid. The product so obtained was boiled with alcohol and filtered from a small quantity of tarry residue. On the addition of water a colourless crystalline powder gradually separated. M.P. indefinite, shrinks and softens about 150°. (Found: C, 41·9; H, 3·69; N, 18·95; H_2O, 1·4 %. $C_{20}H_{19}O_{12}N_8$, 0·5 H_2O requires: C, 42·03; H, 3·51; N, 19·61; H_2O 1·58 %.)

Di-(3 : 5-dinitrobenzoyl)-l-cystine. 0·48 g. of cystine (1 mol.) dissolved in 8 ml. *N* NaOH reacted with 0·93 g. of the acid chloride (2 mols.). On acidifying the alkaline solution with dilute HCl a gummy mass was precipitated which became friable on standing. This was filtered off and purified by dissolving in hot alcohol, filtering and adding water until just turbid. The liquid was then warmed until just clear and allowed to stand. The whole mass set to a gel which was broken up, filtered and dried in a desiccator, giving a powder which gradually turned brown in the course of a few days. Darkened at 154°, melted with decomposition about 180°. (Found: N, 12·41, 12·54; S, 9·38, 9·67 %. $C_{20}H_{16}O_{14}N_6S_2$, $2H_2O$ requires: N, 12·66, S, 9·64 %. Loss of H_2O at 100°, 4·4 %. Cal. 5·1 %. Found for anhydrous substance N, 13·13 %. Calc. 13·37 %.)

The dry powdered product again formed a gel when dissolved in alcohol and diluted with water. A gel was also formed from aqueous acetone, dilute HCl and dilute acetic acid. Insoluble in benzene. The nitroprusside test was given after reduction with Zn and HCl.

Di-(3 : 5-dinitrobenzoyl)guanidine. 0·54 g. of guanidine sulphate was dissolved in 10 ml. *N* NaOH, 1·15 g. of powdered 3 : 5-dinitrobenzoyl chloride added and the mixture shaken for about 2 minutes. The guanidine derivative (insoluble in NaOH) was filtered off and recrystallised from alcohol in radiating clusters of colourless needles. Insoluble in water, moderately soluble in acetone, slightly soluble in benzene: M.P. 280° with blackening (shrinks about 215°). The filtrate from the reaction gave no precipitate with HCl, showing that all the acid chloride had reacted. (Found: N, 22·05 %. $C_{15}H_9O_{10}N_7$ requires: N, 21·93 %.) The substance was hydrolysed by boiling with 1 : 1 HCl for 1 hour. On cooling 3 : 5-dinitrobenzoic acid crystallised out.

Reaction between d-glutamic acid and 3 : 5-dinitrobenzoyl chloride. 0·73 g. (1 mol.) of glutamic acid was dissolved in 15 ml. of *N* NaOH. 1·15 g. (1 mol.) of powdered 3 : 5-dinitrobenzoyl chloride were added and the mixture shaken for 2 minutes. Nothing was precipitated on the addition of dilute acetic acid. Acidification with HCl gave an oil which crystallised on standing. After some time the crystalline precipitate was filtered off and the filtrate made up to 100 ml. It still contained unchanged glutamic acid. Using Sørensen's formaldehyde titration, 10 ml. of this solution required 2·1 ml. of *N*/10 NaOH. This indicated that 42 % of the glutamic acid was unattacked.

The precipitate was dissolved in cold alcohol (in which it was very soluble). Water was added until just cloudy, and the mixture was allowed to stand, whereupon 0·17 g. 3 : 5-dinitrobenzoic acid separated. The alcohol was evaporated from the filtrate and 3 : 5-dinitrobenzoylglutamic acid crystallised out. It was recrystallised from water as colourless needles. The substance softens and shrinks at 98–99°. (Found: C, 41·24; H, 3·26; N, 11·89 %. $C_{12}H_{11}O_9N_3$, 0·5 H_2O requires: C, 41·14; H, 3·28; N, 12·0 %. Loss in weight when dried at 100°, 2·4 %. Calc. 2·6 %. Found for anhydrous substance: N, 12·45 %. Calc. 12·32 %.)

It was subsequently found that a better separation of 3 : 5-dinitrobenzoic acid from the glutamic acid derivative could be obtained as follows. The reaction was carried out as above except that 1·5 g. of sodium acetate were added before the addition of the dinitrobenzoyl chloride. On acidifying with HCl, 3 : 5-dinitrobenzoic acid separated in crystalline form (0·53 g.). The filtrate on standing for a few minutes deposited 3 : 5-dinitrobenzoylglutamic acid (0·54 g.). This was then recrystallised from hot water.

Reaction between aspartic acid and 3 : 5-dinitrobenzoyl chloride. 0·66 g. of aspartic acid was dissolved in 15 ml. of *N* NaOH and 1·15 g. of powdered 3 : 5-dinitrobenzoyl chloride added and the mixture shaken for 2 minutes. No precipitate was produced on acidification with dilute acetic acid, but 0·5 g. 3 : 5-dinitrobenzoic acid separated at once on addition of dilute HCl. Formaldehyde titration showed that 50 % of the aspartic acid remained unchanged.

Separation of glycine from aspartic acid. A mixture of 0·38 g. of glycine and 0·66 g. of aspartic

acid was dissolved in 20 ml. of *N* NaOH. 1·3 g. of 3 : 5-dinitrobenzoyl chloride were added (an excess for the glycine present). The mixture was shaken, filtered and acidified with dilute HCl. The precipitate, which consisted of a mixture of the glycine derivative and 3:5-dinitrobenzoic acid, was dried and shaken with light petroleum-alcohol (5 : 2) mixture in the cold. The residue was recrystallised from water: M.P. 178°. The presence of large quantities of sodium acetate and other sodium salts did not impair the separation.

In the following mixtures the first named component was separated by a method similar to the above: asparagine from aspartic acid; glycine from gelatin (an equal weight); glycine from glutamic acid.

In presence of gelatin an excess of alkali was required. Also the product on acidification was usually oily, but crystallised if allowed to stand for some time. For the mixture of glycine and glutamic acid it is an advantage to have as little excess of 3 : 5-dinitrobenzoyl chloride as possible (calculated for the glycine).

Separation of leucine and glycine. A mixture of 0·33 g. of leucine and 0·19 g. of glycine was dissolved in 10 ml. of *N* NaOH. 1·15 g. of 3 : 5-dinitrobenzoyl chloride were added and the mixture shaken. On acidification with acetic acid 3 : 5-dinitrobenzoylleucine crystallised out. After standing for some time (to ensure complete precipitation) this was filtered off and recrystallised from aqueous alcohol: M.P. 186°.

Dilute HCl was added to the filtrate to precipitate the glycine derivative. Recrystallised from water: M.P. 178–179°.

Reaction between thiolacetic acid and 3 : 5-dinitrobenzoyl chloride. 0·92 g. of thiolacetic acid was dissolved in 20 ml. of *N* NaOH and shaken with 2·30 g. of 3 : 5-dinitrobenzoyl chloride. The nitroprusside test became negative almost immediately. On acidification either with dilute acetic or hydrochloric acid an oil was produced which solidified on long standing. The substance proved difficult to recrystallise. (Found for unrecrystallised substance: N, 10·3; S, 12·0 %. Calc. for $C_9H_{16}O_7N_2S$: N, 9·8; S, 11·19 %.) Melts with decomp. about 135°. Very soluble in alcohol and acetone, slightly soluble in benzene. When recrystallisation was attempted by dissolving in alcohol and adding water, an interchange of radicals evidently took place as the substance which crystallised out contained no sulphur and proved to be ethyl 3 : 5-dinitrobenzoate.

The product of the condensation was undoubtedly *S*-3 : 5-dinitrobenzoylthiolacetic acid. It was completely hydrolysed by boiling with 5*N* HCl for 30 minutes. On cooling 3 : 5-dinitrobenzoic acid separated out, and the filtrate gave a strong nitroprusside reaction.

Reaction between cysteine and 3 : 5-dinitrobenzoyl chloride. 0·79 g. of cysteine hydrochloride (1 mol.) was dissolved in 15 ml. of *N* NaOH and shaken with 1·15 g. (1 mol.) of the acid chloride. The nitroprusside test became negative almost immediately. The solution was filtered and a brown powder precipitated by the addition of dilute acetic acid. The product was purified by dissolving in alkali and reprecipitating with acetic acid. Melted with decomp. about 160°. Very soluble in alcohol and acetone, slightly soluble in water, insoluble in benzene. Hygroscopic. (Found: N, 13·31; S, 9·96 %. $C_{10}H_9O_7N_3S$ requires: N, 13·33; S, 10·1 %.)

On carrying out the reaction with 2 mols. of the acid chloride the same substance appeared to be produced. The reaction was also carried out in an atmosphere of nitrogen with identical results.

SUMMARY.

1. 3 : 5-Dinitrobenzoyl chloride is recommended as a reagent for the rapid identification of many amino-acids, crystalline derivatives usually being obtained in good yield. The condensation is almost instantaneous in alkaline solution.

2. The acidic amino-acids appear to be less reactive towards this reagent. Under the conditions cited in the experimental part aspartic acid does not give a derivative. In this way glycine and several other amino-acids have been separated from aspartic acid.

Although glutamic acid does give a derivative, 3 : 5-dinitrobenzoylglycine is formed preferentially and a separation has been based on this fact.

No condensation could be effected with tyrosine in alkaline solution.

3. The 3 : 5-dinitrobenzoyl derivatives of certain amino-acids are precipitated from alkaline solution by dilute acetic acid. Others are not precipitated by this reagent, but only on the addition of dilute HCl. In this way glycine and leucine have been separated.

4. Guanidine gives a good yield of crystalline di-(3 : 5-dinitrobenzoyl) guanidine, which is insoluble in alkali.

5. Thiolacetic gives *S*-3 : 5-dinitrobenzoylthiolacetic acid by the above method. Cysteine also gives an *S*-derivative which is not, however, suitable for identification purposes.

I wish to thank Sir F. G. Hopkins for the kind interest that he has shown in this work.

REFERENCES.

Buehler, Currier and Lawrence (1933). *Ind. Eng. Chem.* (Anal.) **5**, 277.
Lapworth and Shoesmith (1922). *J. Chem. Soc.* **121**, 1931.
Pfeiffer (1914). *Ber. deutsch. chem. Ges.* **47**, 1581.
Saunders (1933). *Biochem. J.* **26**, 397.
Wertheim (1929). *J. Amer. Chem. Soc.* **51**, 3661.

LXXXIII. THE RELATIONSHIP OF IRON WITH THE AGEING OF CELLS.

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The life of the multicellular organism is divided into certain phases by developmental events which occur during those periods. During embryonic and early post-embryonic existence cell division and cell growth are exceptionally prominent. As cell differentiation increases, cell growth and cell division progressively diminish and finally shrink to a minimum. After the cell has reached its adult stage, it ages and finally dies. Thus growth, differentiation, ageing and death are stages in a continuous process. As these are stages in a process the phases gradually blend into one another.

The results of the following investigation show, for the first time, that a distinct alteration in the chemistry of the cell occurs during development. An examination of the iron content of epithelial organs showed that the cells of an organ in an old animal contain appreciably higher quantities of iron than do those of a young animal. Estimations of copper under similar circumstances did not show such variations.

The iron content of the liver, kidney, testis—and in some cases of the lung and muscle tissue—of rats, guinea-pigs, rabbits, dogs and cats was estimated, all tissue utilised in this investigation being freed from blood before chemical examination by the following methods. Immediately after death a cannula was inserted through the left ventricle into the aorta, and Ringer's solution at body temperature was infused under considerable pressure until the venous fluid was entirely free of blood. Perfusion was carried out in this manner for 20 to 30 minutes at least, and in order completely to exsanguinate the liver and kidney this was followed by further perfusion through the portal vein in the case of the liver. and through the abdominal aorta in the case of the kidney, after all the vessels except the renal artery had been ligatured. The completeness of exsanguination was controlled by histological examination.

The iron determinations were made according to Warburg's [1927] method by which iron may be detected in quantities as small as 5×10^{-4} mg. 50 mg. of thoroughly disintegrated tissue were placed in a platinum crucible and incinerated by heating for 10 to 12 hours at 800° in an electric furnace. 1 ml. of 0·1 *N* HCl was poured into the crucible containing the ash, and 0·1 to 0·2 ml. of the resultant solution was examined for iron. In all, this investigation, which was made at the Charité, Berlin, includes the examination of tissues from 125 animals. The greater number of estimations were on rats; but the study of other animals was sufficient to show that in them the results were similar to those in the rat.

The iron content of pulmonary tissue showed greater variations than that of the liver, kidney and testis. These irregularities may possibly be explained by the inhalation of iron during life. Hence the examination of lung tissue was carried out only in a relatively small number of rats. Similarly the spleen may contain an

appreciable amount of iron as a result of the destruction of erythrocytes, and therefore this organ was excluded.

Is the increase in the iron content of aged cells of special significance or is it dependent on the destruction of blood during life? The latter possibility can be excluded for the following reasons.

1. Histological examination did not show this increased iron to be due to haemosiderosis, and further the increased iron occurred regularly in organs, such as in the testis, in which haemosiderosis does not usually take place.

It might be claimed however that the histochemical methods for the demonstration of iron are too crude to demonstrate the minute amounts present in the cells, or to show variations when these are present.

2. The lower values for iron found in the tissues of young animals and the high values of the old are definite and constant. This is not only true in different animals of the same species but also in animals of various species during corresponding phases of development.

3. If the observed increase in the iron content of the tissues of old animals were due to iron deposited in tissues following blood destruction, then this increase should be continuous throughout life; such is not the case. According to our results the increase in the iron content of the cells in old adults is clearly associated with a definite developmental phase. The increase occurs within a short time and remains at the new level throughout the remainder of the animal's life. This increase of the cell-iron must therefore be of some functional significance.

A study of the iron values of the organs of the rat is illuminating. In the rat, the iron content of the kidney and testis is the same throughout the first 10 months of life (Fig. 1) and in the majority of these animals, even throughout the whole of the first year of life. In a certain proportion of animals, the iron content begins to increase between 12 and 15 months after birth but more frequently after 15 months.

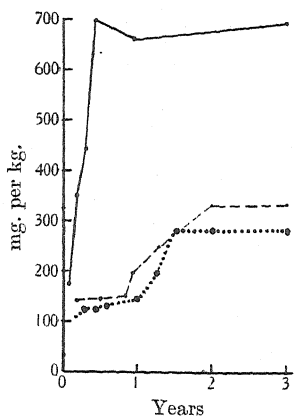

Fig. 1. Rat.

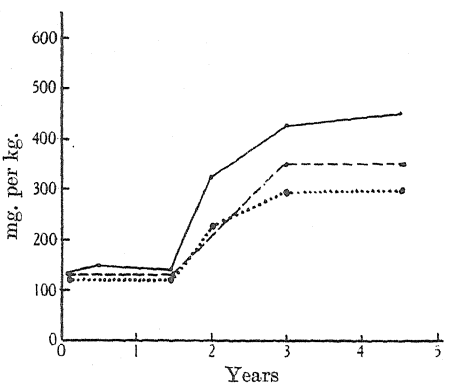

Fig. 2. Guinea-pig.

—— Liver. --- Kidney. • • • Testis.

The values reach their maximum at $1\frac{1}{2}$ years, while only in a small proportion of cases is this higher level delayed until the second year of the animal's life. Throughout the remainder of the rat's existence (in all 3 to 4 years) the iron values remain the same. The increase of iron in the liver occurs at about the third month, and maximum values are obtained by the end of the fifth month.

Thus the change from the low level prevalent in the tissues of the young to the high values obtained in the old animals occurs in 3 to 6 months, a relatively short time. Indeed, this rapid increase occurring as it does at a definite interval indicates that this phenomenon is due to some physiological process and is not the result of such a coincidence as the deposition of haemosiderin in the organs.

A further point is also of importance. The rise in iron values occurred in all the types of animals examined and at the same period, that is the middle period of the animal's life.

Since the duration of life of guinea-pigs and rabbits is longer than that of rats, the increase of tissue-iron does not begin before the end of the second year (guinea-pig) or even later (rabbit) (Fig. 2 and Table I). In the liver the increase

Table I. *Rabbit.*

Iron contents of organs at various periods of life in mg. per kg. dried substance—average values.

	Days				Months		Years			
Age	1	3	28	33	2½	3	1	1½	2	6
Liver	1400	—	—	180	160	—	240	200	200	700
Kidney	500	500	—	150	160	—	160	170	—	350
Testis	—	—	125	—	—	—	120	—	125	260

generally begins somewhat earlier than in the other epithelial organs, but the difference is not as great as in the rat. In dogs and cats of 4 to 5 years of age the low values found in the young are still present, and a definite increase in liver-iron does not occur until the seventh year. In the young animals, the results are closely grouped together (from 100 to 180 mg. per kg. of dried tissues, Tables II and III). As already noted, this is true in animals of various species (Table IV). The maximum values of the old animals show somewhat greater variation, but for the different species the readings fall into a definite group (200 to 500 mg.). In the liver the maximum values may be higher—up to 800 mg. (Tables II, III, IV B).

Table II. *Rats.*

Iron content of kidney of rats (mg. per kg. dried substance).

No. of animal	Under ¾ year (young animals)	No. of animal	Over 1½ years (old animals)
1	170	1	268
2	182	2	200
3	162	3	215
4	90	4	435
5	120	5	476
6	107	6	500
7	115	7	326
8	184	8	300
9	170	9	300
10	148		
11	180		
12	123		
13	115		
14	120		
15	140		
Average	142		335

Table III. *Rat.*

Iron content of testis of rats (mg. per kg. dried substance).

No. of animal	Under $\frac{3}{4}$ year (young animals)	No. of animal	Over $1\frac{1}{2}$ years (old animals)
1	96	1	205
2	115	2	355
3	103	3	330
4	85	4	218
5	135	5	294
6	142	6	272
7	157	7	250
8	135	8	264
9	110	9	526
10	128	10	280
11	180	11	182
12	145	12	260
		13	210
		14	400
		15	335
		16	276
		17	270
		18	410
		19	180
		20	345
		21	200
		22	210
		23	270
Average	130		283

Table IV.

Average iron content of organs of animals (mg. per kg. dried substance).

	Animal	Liver	Kidney	Testis
A. Young animals				
	Rat	180	140	130
	Rabbit	170	155	125
	Guinea-pig	145	140	125
	Cat	200	125	—
	Dog	200	150	115
B. Old animals				
	Rat	About 700	335	280
	Rabbit	Up to 700	400	260
	Guinea-pig	450	350	280
	Cat	Up to 800	—	—
	Dog	Up to 600	—	—

As to the iron content of the organs of young animals, Bunge [1898] found that certain animals, *e.g.* rabbits, are born with a relatively large store of iron. But according to the results of this investigation the tissue-iron of rabbits rapidly decreases in amount during the first weeks of post-embryonic life to the same low level which young animals of other species already have from birth (Table I).

Reports have already been published of the estimation of iron in the organs of animals. Very often differences have been found to exist between different species. For instance, the amounts obtained in mice and rats were much larger than those in guinea-pigs and rabbits. The probable explanation is that for small animals, such as mice and rats, the older ones have been utilised in order to obtain sufficient material, while in the case of larger animals, such as guinea-pigs and rabbits, even the organs of the very young are sufficient for analysis.

The iron values are independent of the food intake of the animals. This has been proved by depriving rats of vitamin B-containing food. In spite of this and the consequent considerable loss of weight, the iron content was always found to correspond with that of other animals at the same period of life.

The iron values in the organs of the young are so constant and the increase during age so regular that it is possible to determine the age group of an animal by iron estimations, preferably of the kidney or testis. Low iron values in the testis of rats—below 160 mg. per g. of dried tissue—definitely indicate that the animal is under $1\frac{1}{2}$ years of age, while quantities above 180 mg. suggest that the animal's age is over 10 months and most likely over 15 months. The intermediate period between 10 and 15 months cannot be determined with certainty because it constitutes the transition from the period of low iron values to that of the high values.

The iron content of the striped muscle of young animals is about the same as that found in epithelial organs, especially in the kidney and testis. From this fact we have to conclude that the iron is present not only in cells but also in the paraplasmatic substances in which muscle tissue is very rich. The rise of the iron content in old animals only occurs in the epithelial organs, not in the striped muscle. Therefore the increase of the iron content is probably due to a new iron compound.

Summary.

1. The iron content of the epithelial organs in the animals investigated in this series (rats, guinea-pigs, rabbits, cats and dogs) is constant.

2. A similar constant iron value is obtained in the various organs of the same animal, in the organs of animals of the same species, and as between the organs of animals of the various species. Only in the liver are the variations appreciable, and even here these variations are relatively small.

3. At a definite period of life and within a short time, the iron content of epithelial organs is increased up to 200 %. The same iron value is then maintained until death.

4. This newly acquired iron is probably a compound other than those already present in the cell and must be associated with the ageing of the cell. Hence the longer the life period of the animal the later does the increase occur. The middle period of life can be considered the period during which the increase in all iron occurs.

5. The iron content of the organs of young animals is so constant and its increase at a definite period is so regular that from the iron content of the organs of an animal, especially the kidney and testis, it is possible to determine the age group of the animal from which the tissue has been taken. The cells of epithelial organs having a high iron content can be considered as in the decline of life.

6. Since the increase in the iron content of organs is so regular a feature, the phenomenon must have some biological significance.

References.

Bunge (1898). Lehrbuch der physiologischen und pathologischen Chemie. (Leipzig.)
Warburg (1927). *Biochem. Z.* **187**, 255.

LXXXIV. THE BIOLOGICAL VALUES OF PROTEINS.

V. THE COMPARATIVE BIOLOGICAL VALUES OF THE PROTEINS OF WHOLE WHEAT, WHOLE MAIZE AND MAIZE GLUTEN, MEASURED BY THE GROWTH OF YOUNG RATS.

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In the previous paper of this series [Boas-Fixsen and Jackson, 1932] experiments were described in which the biological values of the proteins contained in various wheat and maize products were determined by the "balance sheet" method, using adult rats. These experiments revealed no difference in value, for maintenance of nitrogenous equilibrium, between the proteins of whole wheat and whole maize. It was pointed out that this result had an important bearing on the theory attributing pellagra, which in its epidemic form is closely associated with the consumption of maize, to an amino-acid deficiency.

A survey of previous work on the subject suggested that, whereas the proteins of wheat and maize might be of equal value for maintaining nitrogenous equilibrium during a short period, a disparity might be revealed by tests in which the growth of young rats during long periods was the criterion employed. The present paper describes experiments of this nature.

The growth of young rats has been used extensively for determining the relative nutritive values of proteins, and the method has been extended to include longevity and reproduction as criteria, by McCollum *et al.* [1921]. Osborne and Mendel [1916; 1920] and Osborne, Mendel and Ferry [1919] have made a detailed study of proteins using this method and devoted much attention to the precautions necessary in this type of experiment in order to permit of trustworthy deductions. They emphasised the importance for growth of the total food intake apart from the protein intake. They considered that ideal conditions for comparison of the biological values of different proteins were attained when rats of similar weight grew at equal rates and ate about the same amount of similarly constituted diets, the only variable in the diets being the nature and proportion, and hence the amount, of protein ingested. This amount was held to be in inverse proportion to the biological value [Osborne and Mendel, 1916].

Subsequently Osborne and Mendel adopted the value of the ratio $\frac{\text{g. gain in weight}}{\text{g. protein ingested}}$ as a numerical expression of the biological value for any given protein. The value of this ratio was found to increase at first as the proportion

of protein in the diet was raised, reaching a maximum after which it declined. This maximum value, obtained under conditions which favoured the most economical use of the protein, was taken as the most trustworthy estimate of its biological value [Osborne, Mendel and Ferry, 1919]. This method involved a long series of experiments with diets containing different proportions of each protein investigated and, besides being very laborious, did not conform to the ideal conditions set out above, for the total consumption of food was not the same in parallel experiments.

Most investigators who have used this method have calculated the weight increase per g. protein ingested from the results of experiments in which the different proteins were present at the same level in the diets and not necessarily at the level at which the above ratio has the maximum value [see also Osborne and Mendel, 1920].

Mitchell [1924, 1; 1928] has offered an explanation for the fact that, for any given protein, the value of the ratio $\frac{\text{g. gain in weight}}{\text{g. protein ingested}}$ (subsequently referred to in this paper as Ratio X_1) has been found to increase with increasing protein intake. He has suggested that in this expression no account is taken of the protein requirement for maintenance, apart from the requirement for growth, and that the former is a greater proportion of the total intake when this is small; in order to obtain uniformity of intake in comparative experiments, he devised his paired feeding method [Mitchell and Beadles, 1930].

Results reported later in this paper tend to support Mitchell's view and show further that if allowance is made for the protein required for maintenance, the above expression tends to give a constant figure for the biological value for growth of any given protein, irrespective of the intake.

Technique.

The experiments here described were confined to diets containing wheat or maize products as source of protein. Most observations were made with whole wheat or whole yellow maize, a few only being made with white maize for comparison with the yellow. The protein content of the maize was low (from 9 to 11 % on the dry weight) compared with that of the wheat, which was relatively high (16·4 %). It was not therefore possible to construct a diet, within the capacity of the animals' appetite, containing sufficient protein to support normal growth derived from whole maize. To see whether, in any case, normal growth could be obtained on maize proteins, diets were prepared containing the gluten of yellow and white maize at various levels up to 23 %. These glutens are by-products obtained in the manufacture of corn starch and consist of a mixture of maize-glutelin and zein, the water-soluble globulins and a proportion of the glutelin having been washed away [Osborne and Mendel, 1914]. They do not therefore represent true concentrates of maize-proteins.

The provision of the vitamin B complex has always been a difficulty in experiments of this type. In work on cereals reliance has been placed either on the cereals themselves for the source of supply (in which case the results are complicated by their differing contents of these vitamins) or on whole yeast. The drawback of a yeast supplement is the addition by this means of small amounts of extraneous protein of good quality [Osborne and Mendel, 1919; Mitchell, 1924, 3].

Osborne and Mendel used chiefly "protein-free milk", while Hoagland and Snider [1926; 1927] used yeast extracts, all of which supplements contain an appreciable amount of nitrogen. In the present work a dilute acetic acid extract from brewer's yeast was used from which the proteins had been precipitated by boiling [Chick and Roscoe, 1929].

The diets were designed to support a subnormal degree of growth, which should be limited only by the amount of protein present. In this connection it is noteworthy that Osborne and Mendel [1916], in a comparison of caseinogen, lactalbumin and edestin, found that the differences

between the nutritive values of these proteins for growth could not be detected if the amounts present corresponded to more than 12·5 % of the total calories. It is found, however, that with diets which contain a very low percentage of protein, the food intake and growth are poor, and the results are very irregular. A 10 % level of protein (on the dry weight) was therefore chosen for the diets with whole wheat and whole yellow maize; the white maize, however, was so poor in protein that it was not possible to make a diet containing more than 9 % protein.

The few recorded estimations of the biological values of proteins of maize and wheat using the criterion of Osborne and Mendel are summarised in Table I. The values are not consistent, those for whole wheat-proteins vary from 1·1 to 2·1; a value of 1·2 was found for whole maize-protein.

Table I. *Nutritional value of proteins of wheat and maize for supporting growth in young rats.*

Source of protein	% protein in diet	Duration of exp. days	Initial wt. of rats g.	Source of vitamin B complex	No. of rats	Gain in body wt. per g. protein ingested	Observer
Whole wheat	10	30	43	Yeast concentrate	6	2·1	Hoagland and Snider [1927]
	10	60	43		6	1·6	
„	10	70	60–70	Wheat	3	1·3	Osborne and Mendel [1920]
„	8	70	„	„	6	1·1	
„	5	70	„	„	4	1·1	„
„	10	56	35–60	Dried yeast	10	1·7	Morgan [1931]
Whole maize	9	56	35–60	„	4	1·2	„
	9	63	—	Maize (in 3 exps. vit. B extract)	8	1·2	Maynard *et al.* [1923]

Materials used.

Table II gives the description and analysis of the cereal products used as sources of protein. The nitrogen was estimated by the Kjeldahl method and the crude fibre and fat by the methods of Weender and Dormeyer, respectively [see Lunge and Keane, 1911]. The carbohydrate was calculated by difference, and the calorie value of the diet as made up was estimated from these data. The composition of the diets, their water and protein contents and their calorific values are set out in Table III. The diets were fed chiefly in the raw condition, but some observations were made using the whole wheat and whole maize diets after mixing with 40–60 % of water and steaming for one hour.

Table II.

		Composition (% of air-dry weight)							
Material	Description	Water	Total protein (N × 6·25)	Zein*	Ash	Fibre	Fat	Carbohydrate (diff.)	Calories (kg.-cal.) per g. (dry wt.)
1. Whole wheat	Soft, red English	14·3	14·1	—	1·4	1·6	1·9	66·7	3·40
2. Whole yellow maize†	S. American	(1) 14·0 (2) 15·2	(1) 9·3 (2) 9·8	3·3 (34)‡	1·2	1·4	2·8	69·6	3·43
3. Whole white maize	S. African	10·7	8·4	2·6 (32)‡	1·1	1·9	4·1	73·8	3·66
4. Yellow maize gluten	Prepared from S. American yellow maize	8·7	27·0	11·7 (43)‡	0·6	1·3	5·2	57·2	3·92
5. White maize gluten	Prepared from (3)	6·9	41·5	22·0 (53)‡	0·7	0·7	5·6	44·6	3·95

* (N soluble in 90 % alcohol) × 6·25. † Two samples used. ‡ % of total protein.

Table III. *Composition of diets used.*

Cereal	Wheat	Yellow maize	White maize	Yellow maize-gluten				White maize-gluten	
% protein approx.	10	10	9	9	13	17·6	22	14·5	23
Wheat	690	—	—	—	—	—	—	—	—
Yellow maize	—	1058	—	—	—	—	—	—	—
White maize	—	—	1053	—	—	—	—	—	—
Yellow maize-gluten	—	—	—	312	469	624	815	—	—
White maize-gluten	—	—	—	—	—	—	—	333	535
Salts	25	25	25	25	25	25	25	25	25
Butter fat	35	35	35	35	35	35	35	35	35
Cottonseed oil	23	—	—	20	13	6	—	20	12
Corn starch	363	—	—	709	557	409	225	688	483
% water in diets	12·9	(1) 13·3* (2) 14·0*	10·4	12·3	11·1	10·9	10·1	11·9	10·1
% protein in dry diet (N × 6·25)	9·73	(1) 9·77* (2) 10·1*	9·04	9·02	13·0	17·6	22·5	14·5	(1) 23·0 (2) 24·5†
% total cals. from protein	8·90	(1) 9·58* (2) 9·89*	8·65	7·66	11·3	15·5	20·2	12·3	(1) 20·2 (2) 21·5†
Calorie value per g. dry diet	4·37	4·08	4·18	4·71	4·61	4·54	4·46	4·70	4·56

* A sample of maize containing lower percentages of water and protein was used in earlier experiments.
† Diet used for last 10 days of experiments.

Digestibility. The digestibility of the protein in the whole maize and whole wheat diets was determined as follows. Young rats were kept in pairs in metabolism cages and fed on the experimental diet for a preliminary period of 3 days; the faeces were collected during a subsequent period of 4 days and the daily faecal output of nitrogen (N) and the nitrogen intake (I) estimated. The daily faecal output of endogenous nitrogen (E) was also determined for each pair of rats during a period in which a nitrogen-free diet was fed [see Boas-Fixsen, 1930]. The undigested nitrogen of the protein tested is then equal to N − E, and the digestibility to $\left(1-\frac{N-E}{I}\right)\times 100$. The average figures, calculated from four experiments on each diet, were as follows: wheat, raw 84 %, cooked 85 %; maize, raw 92 %, cooked 89 %. Cooking did not therefore appear to alter the digestibility of these proteins, although it increased the food intake (see below, p. 597). The slightly higher values obtained for maize are probably not significant.

Mitchell [1924, 2] also found the proteins of whole maize and whole wheat to be equally digestible, but the value determined, 94 %, was higher.

There is some disagreement among different authorities as to the allowance that should be made for nitrogen of endogenous origin in the faecal nitrogen [see Schneider, 1934]. According to Mitchell [1924, 2], E is proportional to the amount of food taken, and in the expression N − E, the value of E should be adjusted to correspond with the actual food intake in the experiments with the proteins under consideration, which is almost invariably greater than in the trials on nitrogen-free diets. If such an allowance be made in the present case, the digestibility values are slightly raised, and those for the wheat diets slightly more than the corresponding figures for the maize diets, since rather more was eaten of the wheat than of the maize diet. The figures become: wheat-protein, raw 88 %, cooked 91 %; maize-protein, raw 94 %, cooked 92 %.

Experimental.

Black and white rats from the Lister Institute stock were used. At first, litters of young rats were placed on experiment at 21–22 days of age, irrespective of the body weight, which varied from 34 to 54 g. Subsequently it was found more satisfactory to keep the young rats on the ordinary breeding diet until they weighed 50 g. or over. The experiment proper lasted for 10 weeks; the first week was treated as a preliminary period to enable the animals to become accustomed to the diet and calculations were made only on the results of the subsequent 9 weeks. Each rat was kept in a separate cage of the type used in

vitamin B work, a fine wire grid being placed over the coarse wire mesh of the cage floor to prevent loss of food. The legs of the cage rested on a sheet of blotting-paper which covered a tray containing peat. This facilitated the recovery of scattered diet and enabled trustworthy figures to be obtained for the intake of food.

The diets were mixed and stored as air-dry powders. The daily ration was weighed accurately to 0·5 g., added to the residue of the preceding day and mixed with water to a paste. At the end of the experiment, or at the end of any of the periods into which it might be desired to subdivide it, the residue was collected in a weighing-bottle dried at 100° and weighed to 0·1 g.

The diets to be compared were distributed as evenly as possible among littermates. Each rat received, five times a week, a supplement of 4–6 drops (85 mg.) of cod-liver oil to provide vitamins A and D, and 1·5 ml. of yeast fraction "5" as source of the B-vitamins (see above, p. 593); 1 ml. of this extract was derived from 0·5 g. of the yeast, and contained 0·0065 g. nitrogen. The total nitrogen obtained from this source during the 9 weeks period of the observation amounted to 0·46 g.

Results.

The performances of the different groups of rats and a description of their general condition are given in Table IV. In no case was the growth normal, although this standard was approached with diets containing 22–24 % maize-gluten, where the average gain in weight was 100–109 g. in 9 weeks.

Table IV.

	Material	% protein in diet (dry wt.)	No. of rats used	Condition of rats at end of 10 weeks on diet	Increase in wt. g. Variation	Increase in wt. g. Mean	Total food intake (dry) g. Variation	Total food intake (dry) g. Mean	Total caloric intake (mean) kg.-cal.	Protein intake g. (mean)	Ratio X_1 = g. gain in wt./ g. protein intake
1.	Wheat	9·7	14	Good. No long silky hairs in fur	44–66	55	324–510	424	1820	40·5	1·36
2.	Wheat (cooked)	9·7	9	Excellent	42–94	72	364–624	489	2140	47·6	1·51
3.	Yellow maize	9·9	16	Not so good as (1). Coat poorer	32–79	52	327–567	406	1660	40·4	1·29
4.	Yellow maize (cooked)	9·9	9	Good. Coats nearly normal	26–87	65	285–631	471	1920	47·2	1·38
5.	White maize	9·0	6	As group 3	19–85	59	323–628	501	2095	45·3	1·30
6.	Yellow maize-gluten	9·0	4	Poor	(−4)–(+17)	6	176–286	227	1070	20·4	—
7.	,,	13·0	5	Fair	6–78	24	213–285	261	1200	34·0	0·71
8.	,,	17·6	9	Good (Coats nearly normal)	31–74	58	287–505	413	1870	72·6	0·80
9.	,,	22·5	4	Good (Coats nearly normal)	83–128	100	475–666	550	2450	124·0	0·81
10.	White maize-gluten	14·5	7	Stools rather loose	52–77	64	387–520	460	2160	67·0	0·96
11.	,,	23·0*	3	,,	93–125	109	565–674	637	2900	148·0	0·74

* 24·5 for last 10 days.

The amounts of the food intake, protein intake and increase in body weight of rats receiving the whole raw wheat diet (10 % protein, Group 1) are of the same order as those of the animals on the similar diet made with whole raw yellow maize (Group 3). There is a similar correspondence in the results obtained with the cooked wheat and cooked maize diets (Groups 2 and 4). In these experiments, therefore, the values obtained for the biological value of the protein, as estimated by the ratio X_1, are comparable. The value for the protein

of whole raw wheat, 1·32, is slightly higher than for raw yellow maize, 1·29. In the six observations with raw white maize (Group 5, 9 % protein) the average food intake and growth were higher than for Groups 1 and 3; the growth per g. protein was the same (1·30) as for yellow maize.

Of the cooked maize and wheat diets (Groups 4 and 2) more was eaten and there was a larger increase in body weight, also the values of the ratio X were greater than for the raw diets, and that obtained for cooked wheat-proteins (1·51) was definitely larger than for cooked maize-protein (1·38). Since it is unlikely that the nutritive value of the proteins of these cereals is improved by steam cooking, it would appear that the palatability of the diets, apart from the quality of the protein, affected the values for biological value obtained by this method of calculation.

In the experiments with the maize-glutens, a much lower food intake and less growth occurred when the diets contained from 9 to 13 % protein. Only when 14–18 % protein was present did the food intake and weight increase reach the levels (413–460 g. and 58–64 g. respectively) obtained with 10 % protein derived from the whole grains. The amount of protein ingested in the experiments with the maize-gluten was much greater, and the values obtained for the ratio X_1 are correspondingly lower and vary from 0·71 to 0·96 (see Table IV).

Relation between protein intake and weight increase.

In Fig. 1, increase in weight is plotted against protein intake for each rat in the groups receiving diets containing 10 % protein from whole wheat and

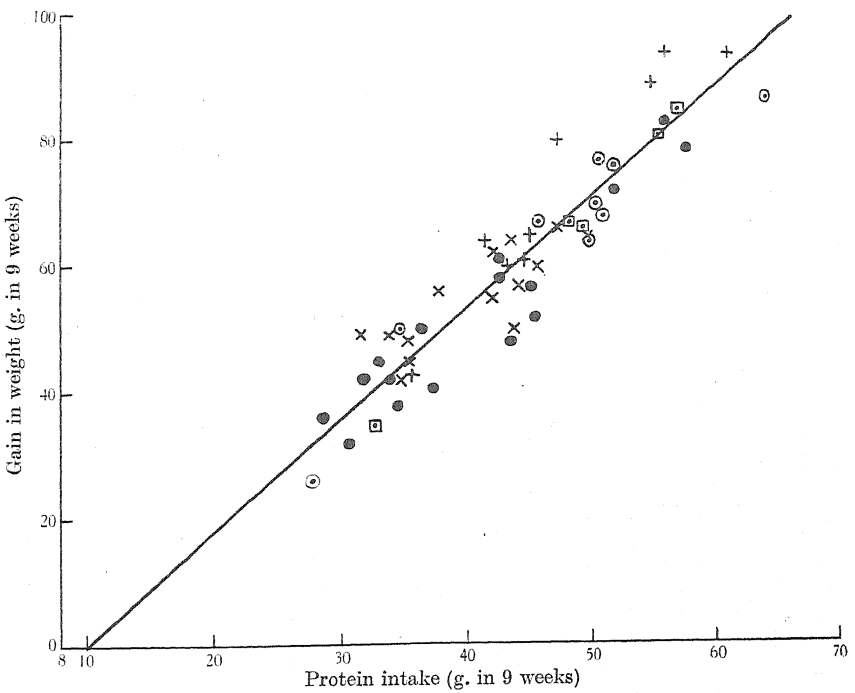

Fig. 1. Relation between gain in weight and protein intake for young rats on diets containing 9–10 % protein from whole wheat and whole maize.

× Wheat, raw. + Wheat, cooked. ● Yellow maize, raw
⊙ Yellow maize, cooked. ⊡ White maize, raw.

whole yellow maize, and 9 % protein from white maize. The experimental points lie grouped about a straight line, which, if produced backwards, cuts the base line at a distance from the origin corresponding to about 10 g. protein. Although it may seem unlikely that 10 g. protein could represent the maintenance requirements for rats whose average body weights varied from 60 to 100 g., yet if this amount is subtracted from the total protein ingested and the excess regarded as being used for growth requirements, the increase in weight is found to be proportional to this excess. The value of the ratio X_2 thus calculated $\left(\frac{\text{g. increase in weight}}{\text{g. protein ingested} - 10}\right)$ was found to be roughly constant in value, irrespective of food or protein intake.

10 g. protein in 9 weeks, together with the nitrogen (0·46 g.) derived from the B-vitamin supplement, provided a daily ration of (25·4 + 7·3) 33 mg. nitrogen, an amount which is about what might be expected for maintenance requirements. Mitchell and Carman [1924] found that about 30 mg. was the daily total nitrogen output on a "N-free" diet of young rats from 60 to 130 g. weight. Using their figure for the biological value of wheat-proteins (av. 67) the daily requirement of wheat-nitrogen to balance this expenditure would be about 44 mg. From the results of experiments with another series of rats, from 45 to 80 g. weight [Mitchell, 1924, 3] the endogenous daily nitrogen requirements can be calculated at from 17 to 23 mg. or about 30 mg. wheat-nitrogen.

Table V. *Biological values of the proteins of whole wheat and whole maize.*

Diet	No. of rats	Caloric intakes	Gain in weight / Prot. intake = (X_1) Variations	Mean	Gain in weight / Prot. intake − 10 = (X_2) Variations	Mean	X_2 mean value
10 % wheat*	7	1415–1694	1·20–1·53	1·36	1·69–2·27	1·91	1·85 ±0·0256
	8	1837–2000	1·14–1·53	1·37	1·48–2·02	1·80	
	8	2026–2727	1·31–1·69	1·50	1·64–2·05	1·87	
10 % yellow maize*	10	1153–1505	0·93–1·45	1·22	1·46–2·03	1·76	1·73 ±0·0229
	6	1713–1846	1·10–1·47	1·31	1·61–1·87	1·69	
	9	2005–2574	1·29–1·52	1·40	1·61–1·90	1·74	
9 % white maize	5	1520–2626	1·07–1·50	1·30	1·44–1·81	—	1·65

* The figures in this Table are derived from all experiments made with these diets, both raw and cooked.

In Table V, which includes the results of all experiments with wheat and maize diets, both cooked and uncooked, values are given of the ratio X_2, obtained after subtraction of this "maintenance protein" from the denominator. Values are also given of ratio X_1, in which this correction is omitted. The data from which these ratios are calculated show great individual variation as regards food intake and gains in weight and the results from the 23 and 25 rats which received respectively the wheat and yellow maize diets are ranged in three groups according to the caloric intake. As this is increased, so the value of X_1 is found to increase, whereas that of X_2 remains approximately constant.

The values for X_2 calculated from experiments with the cooked wheat and maize diets are grouped together with the values derived from the experiments with the raw diets, because they showed no significant difference. The figures were: for wheat-proteins, raw 1·83, cooked 1·90; for maize-proteins, raw 1·72, cooked 1·74. As shown in Table IV, both intake, growth and the values for X_1 were greater for the cooked than for the raw proteins, but since the correction in the denominator, employed in calculating X_2, is proportionally less for the cooked than for the raw diets, the values for X_2 tend to become more nearly

identical than those for X_1 (wheat-proteins, raw 1·36, cooked 1·51; maize-proteins, raw 1·29, cooked 1·38).

The average value of X_2 obtained for whole wheat-protein, 1·85, is slightly higher than that for the whole maize-protein, 1·73. The difference between these mean values (0·12) is more than three times the probable error of this difference (0·0343); there is therefore a more than 20 : 1 chance that the difference found between the nutritive values of wheat- and maize-proteins for growth is significant. On the other hand, in comparison with the maize diet, the wheat diet contained a slightly lower proportion of protein relative to the total calories; and the protein may therefore have been utilised with slightly greater efficiency for that reason.

Maize-gluten. The results in Table IV indicate an inferiority of the maize-gluten diets as compared with those composed of the whole grain. For example, the rats in Group 8, receiving diets containing 17·6 % protein from yellow maize-gluten ingested food of about the same total calorie value (1870 kg.-cal.) and showed about the same average increase in weight (58 g.) as the rats in Group 3, receiving diets with only 10 % protein derived from the whole maize grain (1660 kg.-cal.; increase in weight 52 g.) and as those in Group 1 receiving a similar diet prepared from whole wheat (1820 kg.-cal.; increase in weight 55 g.). In the first case this amount of growth was supported by a total of 73 g. protein whereas in the second and third instances only 40 g. protein were needed.

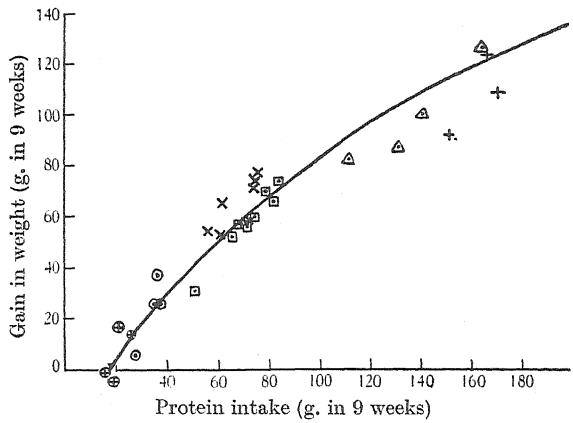

Fig. 2. Relation between gain in weight and protein intake for young rats on diets containing 9 to 23 % protein from maize-gluten.

		% protein
⊕	Yellow maize-gluten	9·0
⊙	,, ,,	13·5
⊡	,, ,,	17·5
△	,, ,,	22·0
×	White maize-gluten	15·0
+	,, ,,	23·0

Fig. 2 shows graphically the results of the whole series of experiments with the yellow and white maize-glutens. In this case the points appear to lie on a flat curve and no simple relation is shown between the weight increase and protein intake. It should be remembered, however, that in the experiments with whole wheat and whole maize illustrated in Fig. 1, the protein was fed throughout at approximately the same level (10 %) whereas, in the experiments

with the glutens, the protein level varied from 9 to 24 %. The form of the curve indicates that the gluten is utilised with less efficiency when present in the diets at higher levels.

The point at which the curve cuts the abscissa corresponds with an intake of about 18 g. protein in 9 weeks, which would suggest that the amount of maize-gluten-protein required for maintenance is greater than the amount required of whole maize-protein.

Since, however, this point for maize-gluten is determined mainly by experiments with diets containing over 14 % protein, this value for maintenance (18 g.) cannot fairly be compared with that obtained for the whole cereal diets (10 g.). The results of the experiments with 9 % protein from yellow maize-gluten appear, at first sight, to indicate that about 15–25 g. maintenance protein are required at this level also. It must, however, be remembered that, in these experiments (Group 6, Table IV), the caloric intake was very low, so that some protein may have been used to provide energy and the protein maintenance requirements raised on that account.

In the samples of white and yellow maize the proteins contained 32 % of zein (calculated from the amount of nitrogen soluble in strong (90 %) alcohol) and presumably about 68 % of glutelins and globulins [see also Osborne and Mendel, 1914]. The similarity in nutritive value between the total proteins of whole wheat and of whole maize indicates that the glutelin and globulins contained in the maize are able to supplement the deficiencies of the zein.

In the specimens of maize-gluten used, the proportion of alcohol-soluble protein was much greater than in the whole grain, and amounted to 43 and 53 % of the total protein in the yellow and white maize-glutens, respectively. Presumably the 57 to 48 % present of other maize-proteins retained in the gluten was insufficient to supplement this larger proportion of zein.

Summary.

1. An attempt has been made to measure the relative nutritive values of the proteins of wheat and maize for the growth of young rats, using the criterion adopted by Osborne and Mendel, *viz.* the gain in weight per g. protein ingested.

2. The proteins of whole wheat and of whole yellow and white maize administered in diets containing 9–10 % of these proteins were found to have nutritive values of about the same order, being 1·36, 1·29 and 1·30 respectively, as determined by the above method. After cooking, these cereal diets were taken better by the animals, and the nutritive value of their proteins appeared to be slightly greater (1·51 and 1·38 for wheat and yellow maize-proteins respectively).

3. Graphic representation of the above results indicated that, if an amount of protein (10 g.) were subtracted from the total protein ingested over the experimental period of 9 weeks, the ratio, growth/g. protein ingested, was roughly constant in value and independent of the food intake. This 10 g. might be supposed to represent "maintenance requirements" apart from those of growth. The value of the ratio $\frac{\text{g. weight increase}}{\text{g. protein ingested} - 10}$ would then indicate the biological value of the proteins for the growth of young rats.

4. The value of this ratio was 1·85 for the proteins of wheat, and 1·73 for those of maize, when present in the diet at a 9–10 % level. There was no difference between values obtained for the cooked and raw cereals.

5. The nutritive value of "maize-gluten" was found to be inferior to that of the proteins of the whole grain, doubtless due to the greater proportion of zein contained.

6. The results of the above experiments, showing only a slight difference between the nutritive values of the proteins of whole wheat and whole maize for support of growth in the young rat, is in accord with previous work showing their similar values for maintenance of the adult rat.

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REFERENCES.

Boas-Fixsen (1930). *Biochem. J.* **24**, 1794.
—— and Jackson (1932). *Biochem. J.* **26**, 1923.
Chick and Roscoe (1929). *Biochem. J.* **23**, 504.
Hoagland and Snider (1926). *J. Agric. Res.* **32**, 679.
—— —— (1927). *J. Agric. Res.* **34**, 297.
Lunge and Keane (1911). Technical methods of chemical analysis, **2**, 447, 451. (Gurney and Jackson, London.)
Maynard, Fronda and Chen (1923). *J. Biol. Chem.* **55**, 145.
McCollum, Simmonds and Parsons (1921). *J. Biol. Chem.* **47**, 111.
Mitchell (1924, 1). *Physiol. Rev.* **4**, 424.
—— (1924, 2). *J. Biol. Chem.* **58**, 873.
—— (1924, 3). *J. Biol. Chem.* **58**, 905.
—— (1928). *Biochem. J.* **22**, 1323.
—— and Beadles (1930). *J. Nutrit.* **2**, 225.
—— and Carman (1924). *J. Biol. Chem.* **60**, 613.
Morgan (1931). *J. Biol. Chem.* **90**, 771.
Osborne and Mendel (1914). *J. Biol. Chem.* **18**, 1.
—— —— (1916). *J. Biol. Chem.* **26**, 1.
—— —— (1919). *J. Biol. Chem.* **38**, 223.
—— —— (1920). *J. Biol. Chem.* **41**, 275.
—— —— and Ferry (1919). *J. Biol. Chem.* **37**, 223.
Schneider (1934). *Biochem. J.* **28**, 360.

LXXXV. BRADYCARDIA IN THE VITAMIN B_1-DEFICIENT RAT AND ITS USE IN VITAMIN B_1 DETERMINATIONS.

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In an earlier paper [Drury *et al.*, 1930] it was shown that rats suffering from vitamin B_1 deficiency had low heart rates. When vitamin B_1 was administered rapid cures resulted, the effect on the heart rate being proportional to the amount of vitamin allowed. It was suggested that this action on the heart might form the basis of a very convenient, rapid and accurate method for estimating the vitamin. Vitamin B_2 and various other food constituents were without similar action. During the past three and a half years this method has been in continuous use in this laboratory where it forms our usual routine method of test in vitamin B_1 assays. As a result of this further experience—during which several hundred tests in all have been carried out—we have had the opportunity of collecting further evidence as to the advantage of the method and have examined its degree of accuracy when carefully checked against other standard methods of vitamin B_1 assay. In the present paper we present a typical set of results, illustrating the use of the method in practice. The principal examples which we give relate to tests on the materials selected for investigation in 1930 by the sub-committee appointed in this country to examine methods and standards for vitamin B_1, preparatory to the adoption of the League of Nations International Standard. These materials were (1) the "activated acid clay" standard, and certain specimens of (2) yeast, (3) marmite and (4) wheat germ. Parallel determinations on each of these four materials were carried out by us by three other methods, *viz.* (1) cure of convulsions in rats, (2) growth rate in rats, and (3) cure of "polyneuritis" (head retractions) in pigeons, and are here compared with our results by the bradycardia method. These data were included in the memorandum on antineuritic vitamin B_1, placed before the International Conference on Vitamin Units and Standards of the Permanent Standards Commission of the Health Organisation of the League of Nations, London 1931.

PART I.

Determination of vitamin B_1 by the bradycardia method.

1. *Technique.*

The electrocardiographic records needed in this method are taken by a procedure already outlined [Drury *et al.*, 1930]. The unanaesthetised rat is held down firmly on its back on a board, its head being held by an adjustable metal clamp and its paws and hind-legs fixed by slip nooses attached to cleats at the

side of the board. The electrodes consist of two small needles, one being placed under the skin of the right fore-leg the other at the lower end of the thorax. These needles are connected to a Matthews's portable electrocardiograph on which permanent photographic records are then taken.

The method of carrying out an assay is as follows. Young rats weighing about 40 g. are placed on a basal diet of

Sugar	60
Arachis oil	15
"Light white casein"	20
Salt mixture	5
Autoclaved marmite (1 hr., 15 lbs., p_H 10) ...	6
Cod-liver oil	1 drop per rat per day.

After about 3 weeks, when the animals are beginning to decline in weight, electrocardiograms are taken. By this time the heart rate should have fallen from

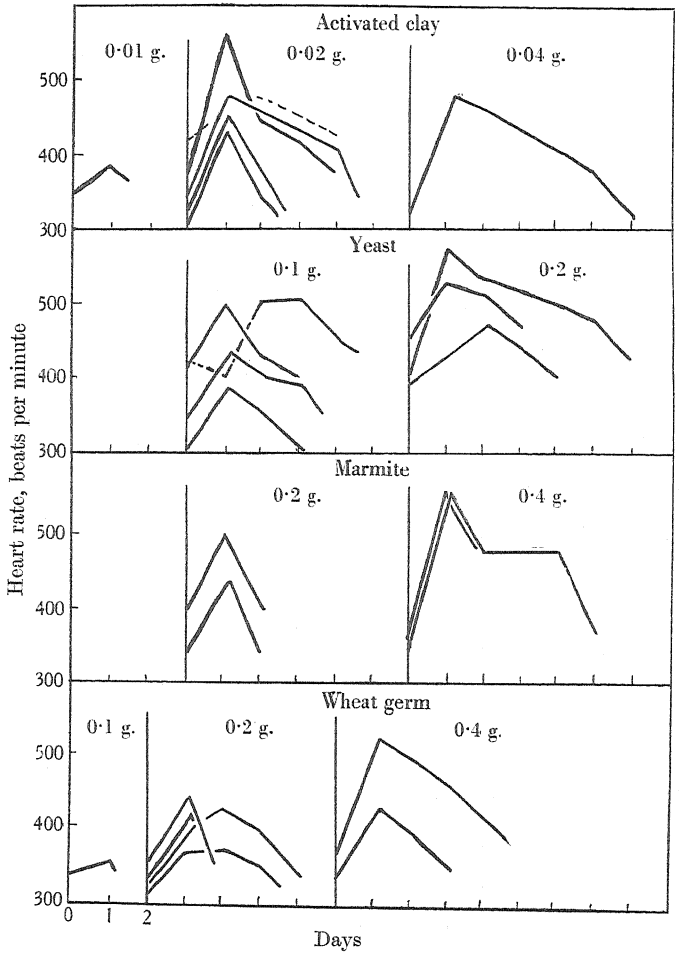

Fig. 1. Heart rate after administration of different sources of vitamin B_1.

Table I. *Duration of cures.*

Material	Dose fed g.	Individual duration of cure (days)	Average duration of cure (days)	Relative activity calculated from curve
Activated clay	0·01	1·5	1·5	
	0·02	2·5, 4·5, 2·5, 4, 4	3·5	100
	0·04	6	6	
Yeast	0·1	3, 3, 3·5, 4·5	3·5	21
	0·2	4, 5, 6	5	16·5
				18·8
Marmite	0·2	2, 2	2	(6)
	0·4	5, 5	5	8·3
				8·3
Wheat germ	0·1	1	1	5·5
	0·2	2, 2, 4, 3·5	3	9
	0·4	5, 3	4	6·5
				7

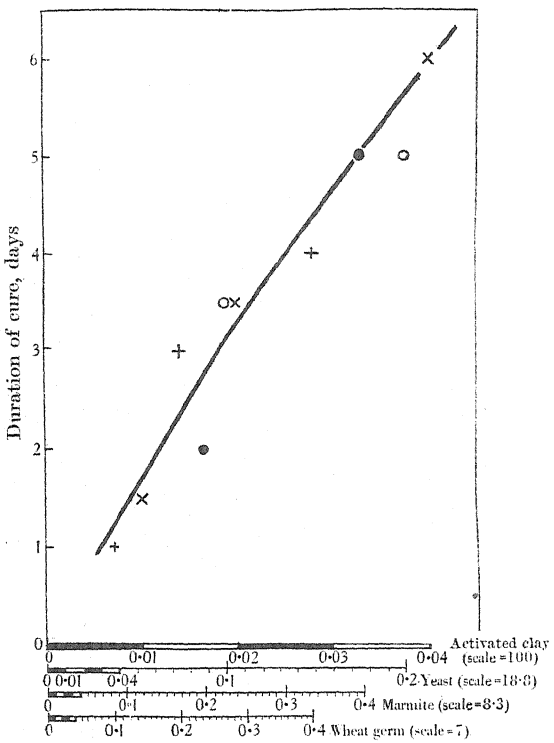

Fig. 2. Bradycardia method: dose-effect curve.

× = Activated acid clay standard.
o = Yeast.
• = Marmite.
+ = Wheat germ.

To determine the scales for the unknown, the points for the standard are first plotted; unknowns are next plotted roughly at approximately the correct scale; and then the value for each point of the unknown is calculated against the standard curve. The average of these values (shown in Table I) is used in re-plotting for final comparison, as above.

the normal rate of 500–550 per minute to a rate of about 350 per minute. As soon as the rate has fallen to this figure a single dose of the substance to be tested is fed. The animal is replaced in its cage and given basal diet and water *ad lib.* as before. After 24 hours its electrocardiogram is again taken. If the rhythm has not increased a larger dose must be tried. With a sufficiently large dose the rate is suitably increased and is measured every 24 hours, or better twice daily, until it has fallen again to about the same low value as when the dose was administered. The increase in rate and the time over which the increase lasts are roughly proportional to the dose given. Several levels of unknown are tested in this way, with about 4 to 6 animals in each group, and comparison is made simultaneously with the amount of standard reference material needed to produce the same effect. A dose-effect curve may be plotted from which relative activities may be determined for intermediate readings, whenever the "effects" are not quite identical (*cf.* Fig. 2). Cures of about 4 days' duration are found to be the most suitable basis for comparison. A quick method, when approximate results suffice, is to find the amount of supplement needed to give the smallest detectable effect on the heart rate lasting about 1–2 days. If doses lasting longer than about 4–5 days are given errors may be caused by apparently incomplete utilisation of the vitamin. This is especially the case with sources of moderate activity and less so with active concentrates.

2. *Illustrative results.*

In the tests taken for description here the "activated clay" standard was compared with yeast, marmite and wheat germ fed simultaneously at two or three different levels of activity. Fig. 1 gives the daily records of the heart rates after dosing. It is seen that 0·02 g. of activated clay is roughly equivalent to 0·1 g. of the yeast, or slightly more potent than 0·2 g. of marmite or of wheat germ, and other doses in proportion. The average durations of cure at each level of dosage are worked out in Table I, and the relative activities of the four substances can then be calculated more accurately from the combined dose-effect curve, Fig. 2. The activities so determined, taking activated clay as 100, are: yeast 19, marmite 8, wheat germ 7. These results will be found to agree excellently (Table VII) with those determined by the three other standard methods. We now turn to a discussion of the latter, giving various recommendations as to the technique which we have found most advantageous, and shall return later to a comparative discussion of the bradycardia method.

PART II.

Cure of convulsions in rats.

1. *Method.*

Our technique is a modification of that described by Smith [1930].

Diet. The basal diet consists of

"Light white casein"	18
Salt mixture	4
Arachis oil	10
Sucrose	58
Autoclaved brewer's yeast	10
Dried brewer's yeast	0·4
Cod-liver oil	1 drop per rat per day.

The small addition of brewer's yeast is made because in our experience a larger percentage of the rats develop convulsions consistently when a trace of vitamin B_1 is provided in the diet, which is in conformity with the conclusions of Sherman and Sandels [1931] and Sebrell and Elvove [1931]. The 0·4 % level was chosen because after numerous trials it was found to give the best results. Sugar is used in place of starch in order to avoid risk of refection and also because we found that the substitution tended to increase the percentage incidence of convulsions. On this diet over 50 % of animals can be relied upon to develop convulsions at the regular time.

Preparation of animals. Young rats, either albino or piebald, weighing about 70–80 g. are placed on the above diet. After about 6–8 weeks, appetite having fallen considerably, "polyneuritic" symptoms begin to appear. The rat develops leg weakness and drags his limbs, the gait is "wobbly" and there is evidence of inco-ordination (swollen red paws said to be indicative of deficiency of Reader's "vitamin B_4" have not been seen). As a criterion for the presence or absence of definite "polyneuritis" the rat is picked up and suspended by the tail, and rotated between the thumb and finger for a second or two: if convulsions follow the rat is ready for use.

Method of test. A single dose of the substance to be tested is administered to each of a series of such rats. If, as in the work under description, the substance is a foodstuff, it is fed *per os.* If it is an active concentrate it is probably better to inject it into the peritoneum. Provided a sufficient dose has been given the animal is cured within 24 hours. If it is not cured in 2 days the test is regarded as negative, and a larger dose is then given. Animals which die within 24 hours of dosing are neglected, as when they are in the proper condition they should live for at least two days with the "polyneuritic" symptoms. If the dose has been sufficient and the animal is cured, the number of days' respite from convulsions is noted.

Normally, using the minimum dose of standard needed to effect a proper cure, the cure lasts about 5–6 days. With certain substances, however, such as marmite it tends to last slightly longer, *e.g.* about 8–12 days: this may be due to the slower rate of absorption.

Calculation of results. The agreement between duplicate animals as regards the duration of cure is very close. For example a 6-day dose will produce cures lasting somewhere between 4 and 9 days in the great majority of animals in any given group. Occasionally, however, an animal may be encountered which behaves abnormally, appearing to remain cured or partially cured over a protracted period: such animals are neglected in computing the results of the assay. In a few cases these same animals have also failed to respond normally during the depletion period, and coprophagy was suspected—it cannot always be obviated even with screened floors.

If an approximate idea only is desired of the potency of different preparations under test, it can be obtained with great ease and using very few rats by simply comparing the minimum doses of each needed to produce a definite cure—such cures usually lasting about 5 days. This method is especially useful in determining roughly the activity of solutions during a process of concentration.

At about this minimum level, however, results tend to be more variable and partial cures are more frequent. More precise results are obtained therefore by providing sufficient of the supplement to give cures lasting from about 6 to 12 days and feeding several dosage levels of both unknown and standard, using several duplicate rats at each level. Doses of standard and unknown are then compared which give approximately the same duration of response. To allow for slight

differences a dose-effect curve (Fig. 3) is plotted, but it is always advisable to restrict comparisons to doses of unknown and standard which give similar magnitudes of cure. At medium levels the duration of cure is found to be very nearly proportional to the weight of vitamin given, so that the method of calculating activity from "day-dose" (dose given/average duration of cure) is reasonably accurate. As higher levels are reached however the increase in duration of cure does not quite keep pace with the increase in dose.

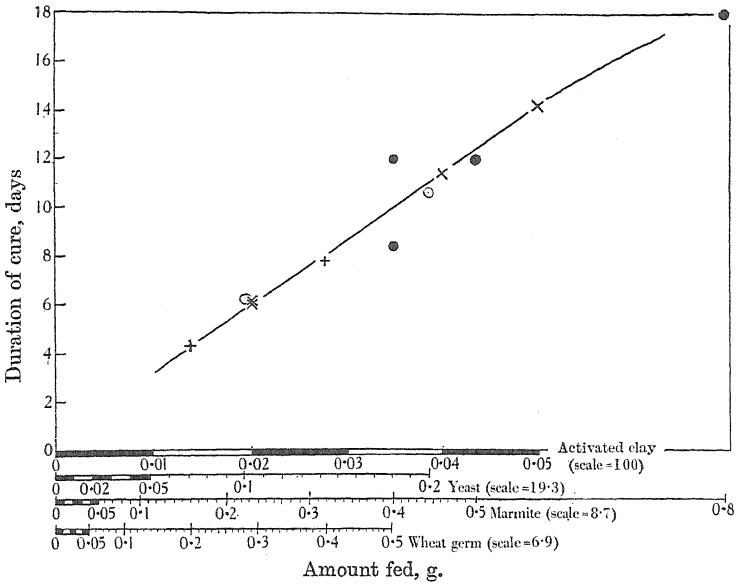

Fig. 3. Convulsion in rats: dose-effect curve.

× = Activated acid clay standard. ○ = Yeast.
● = Marmite. + = Wheat germ.

Scale divisions determined as for Fig. 2.

Degree of accuracy attainable. The relatively small degree of variation between individual animals on duplicate doses of a supplement is seen from reference to Table II. Thus at the level of 0·02 g. of activated clay, 13 out of 20 animals gave cures lasting 4 to 8 days, *i.e.* within 30 % of the median value of 6 days. Or again, with 0·4 g. of marmite 14 animals out of 22 gave a result within 20 % of the median value of 12 days, while 18 out of the 22 fell within 8–16 days, or 30 % of the median. Hence with 5 animals in a group, duplicate determinations based on the median value should certainly not differ by more than 30 %. From a practical point of view this may be taken as the "possible percentage error of the method," *i.e.* when comparing one single dose level of standard with the equivalent level of control and using 5 animals in each group. Actually, in any complete assay based on a series of such comparisons, the final error is naturally considerably less, as will be seen from Tables II and VII.

2. *Results.*

The determinations on yeast, marmite and wheat germ, taking activated clay as standard, are shown in Table II. They confirm the results given by our bradycardia method, the two sets of values being in excellent agreement.

Table II. *Cure of convulsions. Experimental results.*

Material	Dose g.	No. of tests	Days protected	Days protected, mean	Activity calculated from curve
Activated acid clay	0·01	6	(–), (–), (–), (–), (–), (–)	—	—
	0·02	13	10, 7, 6, 4, 4, 4, (20), 8, 7, 5, 7, (13), 5	6·1	—
		[7]	[5, 5, 9, 6, 11, (–), (–)]	[6]	100*
	0·04	4	13, 9, 11, 13	11·5	—
	0·05	3	17, 12, 14		
Yeast	0·05	6	(–), (–), (–), (–), (–), (–)	—	—
	0·10	9	5, 7, 5, 7, 10, 6, 6, 6, 4	6·2	20
	0·20	5	16, 8, 9, 12, 8	10·6	18·5
					19·3
Marmite	0·2	6	(–), (–), (–), (–), (–), (–)	—	—
	0·3	1	(–)	—	—
	0·4	7	10, 7, 7, 10, 6, 11, 8	8·4	7·3
		[15]	[13, 12, 14, 12, 11, 8, 12, 13, 17, 11, 12, 16, 11, 13, 22]	[12·5]	10·5
	0·5	2 [1]	[13], 12, 11	12·0	8·4
	0·8	[1]	[18]	—	—
					8·7
Wheat germ	0·1	3	(–)	—	—
	0·2	5	3, 3, 7, (–), (–)	4·3	7
	0·4	4	8, 6, 10, 7	7·8	6·8
					6·9

Figures in round brackets indicate partial cures, or abnormal response.
Figures in square brackets were determined at a separate time.
*=Standard of reference.

PART III.

Determinations by the growth rate method.

1. *Technique.*

Diet. We use a basal diet having the following composition:

Arachis oil	15
Sugar	60
"Light white casein"	20
Salt mixture	5
Alkaline autoclaved marmite after Guha and Drummond[1]	0·75 g. per rat per day
Cod-liver oil	1 drop per rat per day.

Experiments have also been carried out using rice starch in place of sucrose, and these gave identical results. In order to avoid risk of refection however we prefer to use sucrose, which has the additional advantage of being a more standard product. The "light white" variety of caseinogen is used in order to avoid any possibility of shortage of the "Coward factor".

Influence of variations in vitamin B_2 supplement. In one series of experiments 0·5 g. of autoclaved marmite was given per day in place of 0·75 g. Growth rate was consistently less. This experience emphasises the necessity, frequently overlooked, of always making comparison between animals receiving the same amount of vitamin B_2, in computing results.

[1] Made alkaline to litmus and autoclaved for 1 hour at 15 lbs.

Method of test. During the preliminary period albino rats of 50 to 70 g. weight are fed on the above diet. If smaller animals, around 40 g., are taken lower growth rates are obtained, and results are liable to be more variable. Groups should contain equal proportions of male and female, or preferably males only should be used. As soon as the animals begin to lose weight or are remaining constant, graded daily doses of unknown and standard are fed to groups of rats. The growth rate is then measured over a period of 2–3 weeks.

Calculation of results. Comparison between standard and unknown must be made over that range where small additions of the vitamin have the greatest effect on the growth rate, that is to say between 1 and 2·5 g. per day (Fig. 4). Over

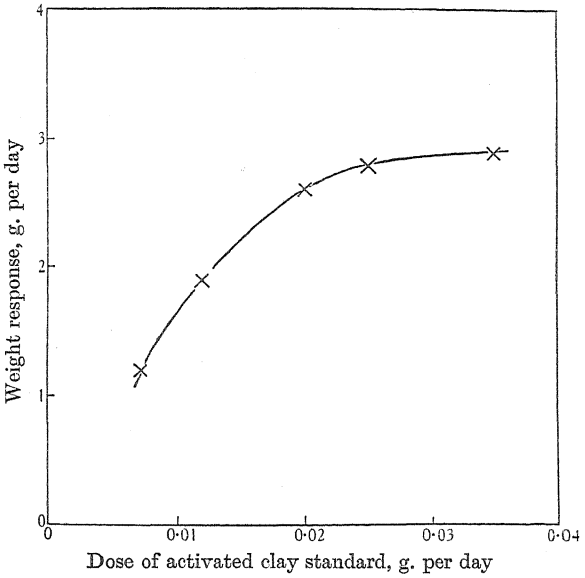

Fig. 4. Growth rate method. Dose-effect curve with international standard (0·75 g. autoclaved marmite per rat per day).

this range the dose is nearly proportional to the growth rate, so that it is legitimate to calculate activity by dividing average gain in weight by dose given. It is preferable however to plot a dose-effect curve for unknown and standard. This should include at least two points for the standard on the steep part of the curve and at least one intermediate or coincident value for the unknown (or *vice versa*).

Variability of "standard reference curve." The suggestion has been made that trouble may be saved and less animals used by constructing a standard reference curve, showing the relation between dose and effect, which, it is suggested, may then be applied to any future experiment. We have found (as we reported in the memorandum to the Vitamin Conference in 1931) that this procedure is inaccurate, since the curve may vary appreciably from one experiment to another, owing to the considerable number of uncontrollable variables. Thus, in one experiment, summarised in Table III (and Fig. 4), in which five levels of standard were tested, using 4 or 5 rats, all males, in each group, the growth was found to be proportional to the dose given up to the level of about 2·5 g. per day and then fell off rapidly, whereas in the earlier experiment (Fig. 6, top), it remained proportional up to 3·5 or over. It follows that direct comparison between

Table III. *Growth responses with graded doses.*

Amount of standard fed mg. per day	Average gain in wt. g. per day	Mean gain in wt.
7	1·2, 1·7, 1·0, 1·1, 1·2	1·2
12	2·5, 2·0, 1·5, 1·9	1·9
20	2·5, 2·7, 2·4, 2·9, 2·6	2·6
25	3·1, 2·8, 2·7, 2·8, 3·1	2·8
35	3·0, 3·2, 2·6, 2·9	2·9

unknown and standard must be made in each individual experiment. This has since been accepted by Coward *et al.* [1933] in a recent paper.

Degree of accuracy attainable. Differences of ±20 % in activity can generally be readily detected by the method described. This is well illustrated by the experiment (Table IV) in which we fed supplements at 80, 100 and 120 mg. per day of marmite to groups of 4 to 5 animals only. Comparing groups of the same sex the differences in response are quite marked (*cf.* dose-response curve shown in

Table IV.

Amount of marmite fed per day mg.	Average daily gains in wt. (g.) ♂	Average daily gains in wt. (g.) ♀	Mean gains in wt. (g.) ♂	Mean gains in wt. (g.) ♀
80	0·44, 0·44	0·16, 0·12, 0·02	0·44	0·10
100	0·82, 0·82	0·24, 0·62	0·82	0·43
120	1·1, 1·3	0·45, 0·70	1·2	0·57

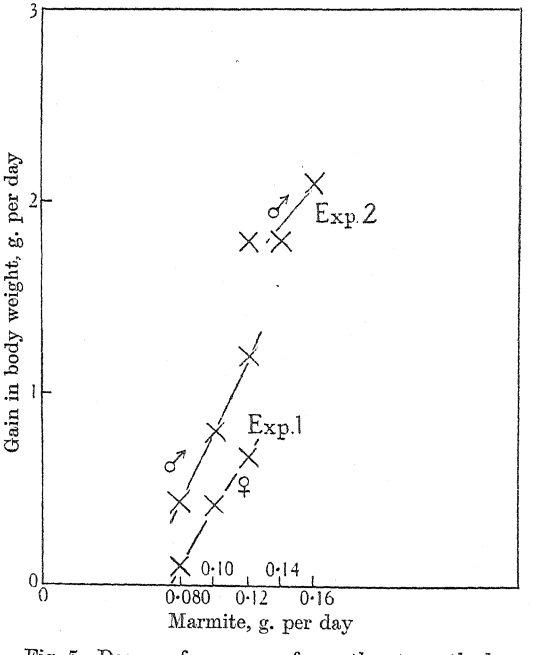

Fig. 5. Degree of accuracy of growth rate method.

Fig. 5). (In this experiment the animals grew at a somewhat slower rate than is usual for this level of marmite. This was attributed to their having started on experiment at the weight of 40 g. in place of the usual 50–70 g.)

To determine the largest individual variation in a larger group of animals and so obtain a more exact measure of the accuracy of the method, a further experiment was carried out. Doses of 160, 140 and 120 mg. of marmite respectively were fed to groups of 9, 5 and 5 animals, all males. The growth rates are given in Table V. It is seen that in the group of 9 the maximum variation is from 1·7 to

Table V. *Individual variability of animals in groups.*

Amount of marmite fed mg. per day	Average daily gains in wt. (g.)	Mean daily gain in wt. (g.)
120	0·9, 2·4, 1·8, 1·9, 1·9	1·8
140	1·8, 1·4, 1·9, 1·0, 1·8	1·8
160	2·4, 1·9, 2·3, 2·4, 2·6, 1·7, 2·0, 1·8, 1·9	2·1

2·6 g. per day, that is about 25 % either way of the median value of 2·1 g. per day. The remaining 7 animals are all within 14 % of the median. Four out of the 9 are within 10 %. In the two groups of 5, 3 or 4 animals agreed within 10 % of the median.

From the foregoing figures it may be expected that in a determination using 5 animals in a group (at a suitable level of dosage), 3 out of 5 animals should agree within 14 %; and an accuracy of this order is about the best that may reasonably be looked for. Actually in the experiment under discussion the difference between doses of 140 mg. and 120 mg. (= −14 %) was not detected when groups of 5 animals were used, but the difference between 140 and 160 mg. (= −12 %) was detected when groups of 5 and 9 animals respectively were used. On the

Table VI. *Growth method. Summary of results.*

Material	Dose (g.)	Average growth rate g. per day	Mean growth rate g. per day	Relative activities calculated from curve
1st series: 0·5 g. autoclaved marmite				
Activated clay	0·007	−1, −0·5	−0·75	
	0·014	2, 1·9, 1·7	1·87	100 (*a*)
	0·028	2·7, 2·2	2·45	
Yeast	0·04	0, 0	0	—
	0·08	1·7, 1·9, 2·6	2·07	20
	0·16	3, 2·4	2·70	21
				20·5
Marmite	0·1	−0·2, 0·5	0·15	—
2nd series: 0·75 g. autoclaved marmite				
Activated clay	0·03	4·2, 3·8	4	(103)
Yeast	0·05	1·8, 1·0	1·40	20
	0·10	3·3, 1·9	2·60	20
	0·20	3·9, 3·7, 3·3	3·63	—
				20 (*b*)
Marmite	0·07	0·75, 0·6	0·68	9·1
	0·14	2·4, 1·5, 1·6	1·83	9·6
	0·28	2·5, 3·4, 3·3	3·07	8·6
				9·1
Wheat germ	0·1	1·2, 0	0·60	6·5
	0·2	1·7, 2·3	2·00	7·5
	0·4	4·2, 3·6, 3·8	3·87	7·5
				7·2

(*a*) = Standard. (*b*) = Secondary standard.

other hand it should be pointed out that when an assay of an unknown is carried out by feeding several different levels of unknown and of standard, the final result

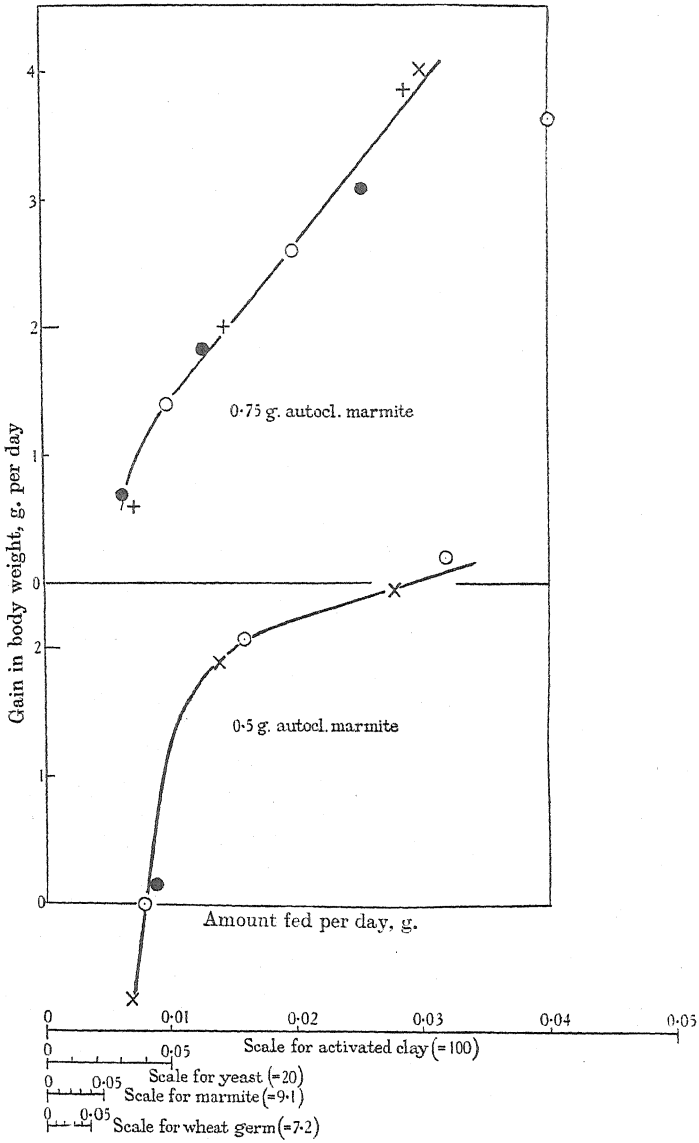

Fig. 6. Vitamin B_1 determination by growth rate method.
× = Activated clay standard. ○ = Yeast.
● = Marmite. + = Wheat germ.

Scale for abscissae determined as in Fig. 2. Note difference in response with 0·5 g. autoclaved marmite per rat per day (below) and 0·75 g. (above).

is then more accurate than just indicated, for although there is a possible error of 14–20 % in each single group, these tend to cancel each other when the final averages are determined (see Table VII).

2. *Results.*

Determinations on activated clay, yeast, marmite and wheat germ gave results virtually identical with those determined by the bradycardia method. Two determinations were carried out: in the first the yeast was compared directly with the acid clay as standard; in the second yeast was used as secondary or derived standard for determining the activity of the marmite and the wheat germ (Table VI and Fig. 6).

PART IV.

Comparison of the "bradycardia" and other methods of assay.

Specificity of test. The results described in Sections I, II and III show that the "bradycardia method" gave identical values with the two other methods of assay, when checked against four materials of diverse origin having activities ranging from 7 to 100. The same figures were given in a few pigeon tests which we also carried out (cure of head retractions)[1]. The comparison of these results, together with further values obtained independently in other laboratories, is given in Table VII. The values obtained by the three rat methods appear to agree within an experimental error of $\pm 5\,\%$.

Table VII. *Agreement between bradycardia and other methods.*

	Relative activities			
Method	Activated clay standard	Dried yeast	Marmite	Wheat germ
Bradycardia method	100	18·8	8·3	7
Cure of convulsions in rats	100	19·3	8·7	6·9
Curative growth tests on rats	100	20	9·1	7·2
Pigeon method	100	18	8·5	—
"Institute B" (rat growth tests)	100	(15)	9	7
"Institute C" (pigeon tests)	100	20	(11)	(14)

Values in brackets are somewhat divergent from five other concordant readings on the same material and are considered less reliable.

This very close agreement (which is confirmed by tests on vitamin B_1 concentrates and other materials) indicates that the factor measured by the bradycardia method runs parallel in its distribution with that measured by the other

[1] The basal diet used was polished rice and water. Only such pigeons as developed head retraction within 30 days were used. These amounted to about 30 % only of the total. Minimum doses were compared giving approximately the same duration of cure and corrected for the "day-dose." Our experience confirms that of other workers that the pigeon gives more erratic results than the rat owing to the greater variation in individual responses. Among the disadvantages of the pigeon method are: (1) a frequent failure to absorb the vitamin properly, especially with materials of only moderate activity, and the occurrence of a "crop bound" condition; (2) an inability to consume many types of foodstuffs of relatively low potency, but of great practical dietetic importance (which of course can be readily tested by the rat method); (3) false cures (*e.g.* as with glucose, change in temperature, *etc.*); (4) lack of economy in use—*e.g.* (*a*) the space needed to house 1 pigeon will accommodate about 10–20 rats, and (*b*) each pigeon is available for test only once in every 6 weeks, whereas with "bradycardia" or "convulsions" tests on a rat several tests per fortnight may be carried out per rat.

better known methods of assay, and it seems very probable that the same single factor is responsible for the loss in weight, for the production of the polyneuritis and for the bradycardia. Physiological arguments in favour of this view are presented in a later section of the paper (Part VI). We have found also that highly purified specimens of vitamin B_1, including the crystalline preparations of Jansen and of Windaus, retain the characteristic effect on the heart beat. At the same time we recognise that in view of the present complicated position of the vitamin B problem it would be premature to make any dogmatic claim on this point.

Advantage of the bradycardia method. The bradycardia method has many advantages over the other methods. All animals are ready for test about 4 weeks after having been placed on the deficient diet, and all consistently develop the symptom. This may be contrasted with the delay and irregularity in the production of head retractions in pigeons or convulsions in rats. Again, results are obtained very rapidly and with little trouble: only one dose has to be fed to each rat and the test is concluded in a few days, one measurement only per day being needed and taking but a few moments to carry out. Also much less material is needed than for a growth test. An additional economy is that the same animal can generally be used two or three times over. The sensitivity of the method is similar to that of the others, although it is probably slightly inferior in this respect to the growth rate method, which however is more laborious and depends on a less specific symptom. A further advantage, and a very important one from the practical standpoint, is that the heart rate method can be used for testing foodstuffs containing only slight traces of the vitamin. Neither the curative pigeon test nor the rat convulsion test is applicable to this important class of estimation, as it is impossible to get the severely ill animal to eat sufficiently large quantities of the food, as for example when the diet must consist entirely of the food in question. (The bradycardia develops at an earlier stage than the polyneuritic symptoms and while the animal is still able to eat normally.) Indeed in certain estimations of this kind, such as in comparisons of white and brown breads and of certain wheat products we have been driven back to the heart rate method as the only one available, since in growth tests on rats we were defeated by the further complication of refection so that no comparison was possible. In the heart rate method an assay can be concluded in 3 or 4 days before refection has had time to develop. It is in this type of problem, *i.e.* comparing the vitamin B activity of naturally occurring and especially farinaceous materials, that the heart rate method seems particularly useful, and we have accordingly added experimental details (Part V) of its application in certain such cases.

Practically the only drawback to the heart rate method is the original expense of purchasing the electrocardiograph. Accurate determinations are not possible with simpler types of apparatus.

PART V.

Application of bradycardia method to wheat products.

To illustrate the practical uses of the bradycardia method, its application to the determination of the vitamin B_1 content of two classes of wheat products will be described. These were (*a*) white compared with brown bread, (*b*) whole wheat grain as influenced by different soil treatments.

(a) White compared with brown bread.

We originally became interested in this problem because it had been claimed that white bread contains adequate amounts of vitamin B [Hartwell, 1924] and that the substitution of brown for white bread in a dietary experiment on rats resulted in little improvement in growth [Mottram and Hartwell, 1929].

Our attempts to compare the vitamin contents of white and brown bread by the rat growth method failed, as the rats became refected. Efforts were made to apply the rat convulsions method, but with unsatisfactory results. Determinations by the bradycardia method were then carried out, and these proved the striking difference between the two kinds of bread.

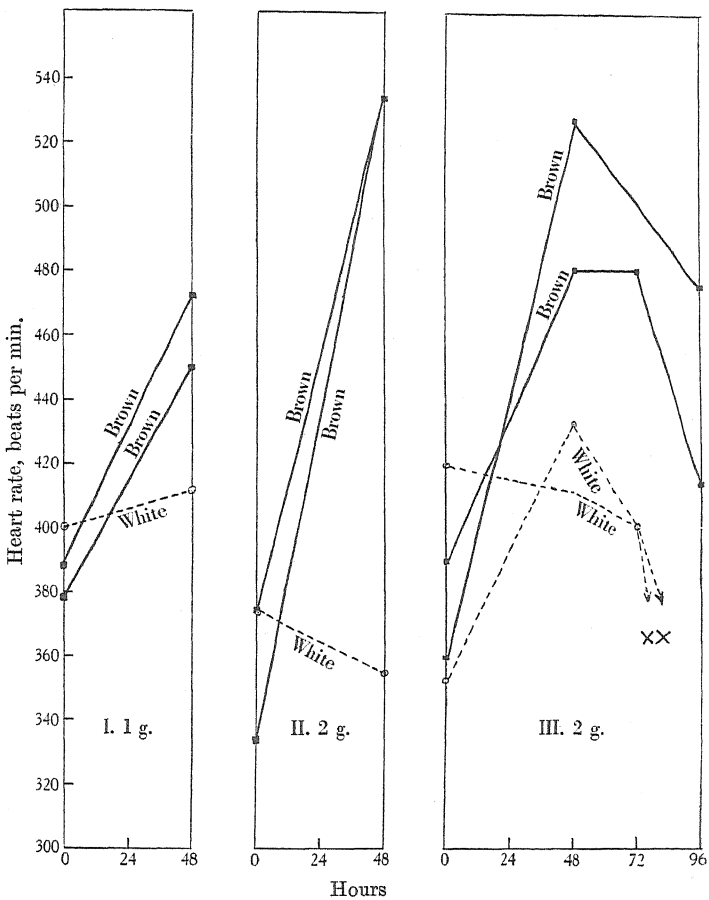

Fig. 7. Comparison of white and brown bread by the heart rate method.

Three tests were carried out. In each case the specimens of brown and white bread were purchased from a local branch of the Co-operative Society. The first test (Fig. 7, I) showed that 1 g. of brown bread was sufficient to cause a marked rise in heart rate whereas 1 g. of white bread had a very slight effect only. The difference was brought out more sharply when the brown bread was fed at a

higher level (Fig. 7, II) and a further experiment (Fig. 7, III) furnishes the basis for a rough quantitative comparison.

Conclusion. The white bread contained only insignificant amounts of vitamin B_1 compared with the brown bread. Detailed results on different types of wholemeal, brown and white and germ breads will be presented in a later paper.

(*b*) *Effect of soil treatment on vitamin value of wheat.*

This work was undertaken to test the claim that the vitamin content of seeds and grain is influenced by the nature of the manure or artificial fertiliser [McCarrison and Viswanath, 1926; Rowlands and Wilkinson, 1930].

Through the co-operation of Sir John Russell of the Rothamsted Experimental Station we were provided with specimens of whole wheat from plots treated as follows:

Plot No. 3. No manure.
2B. 14 tons of dung per acre.
5. Complete mineral manure.
7. Complete mineral manure +412 lbs. of sulphate of ammonia per acre.
10. 412 lbs. of sulphate of ammonia only.

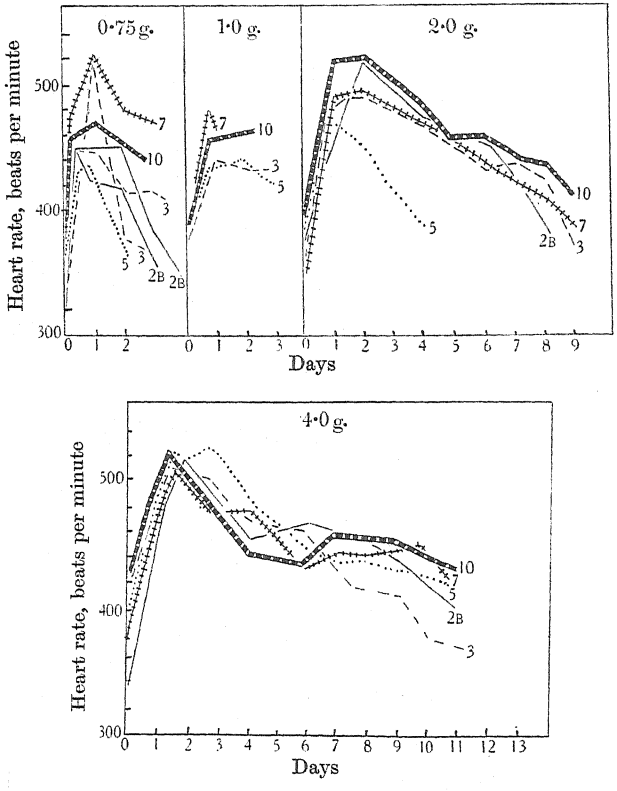

Fig. 8*a*. Vitamin B_1 value of whole meal wheat flours. Preliminary test at various levels (illustrating extent of variation of individual animals).

———— = Plot 2B. — — — = Plot 3. ····· = Plot 5.
+++++++ = Plot 7. ▬▬ = Plot 10.

Although these various treatments greatly influence the chemical composition of the grain and the amount of grain produced our results (Figs. 8*a* and 8*b*) show

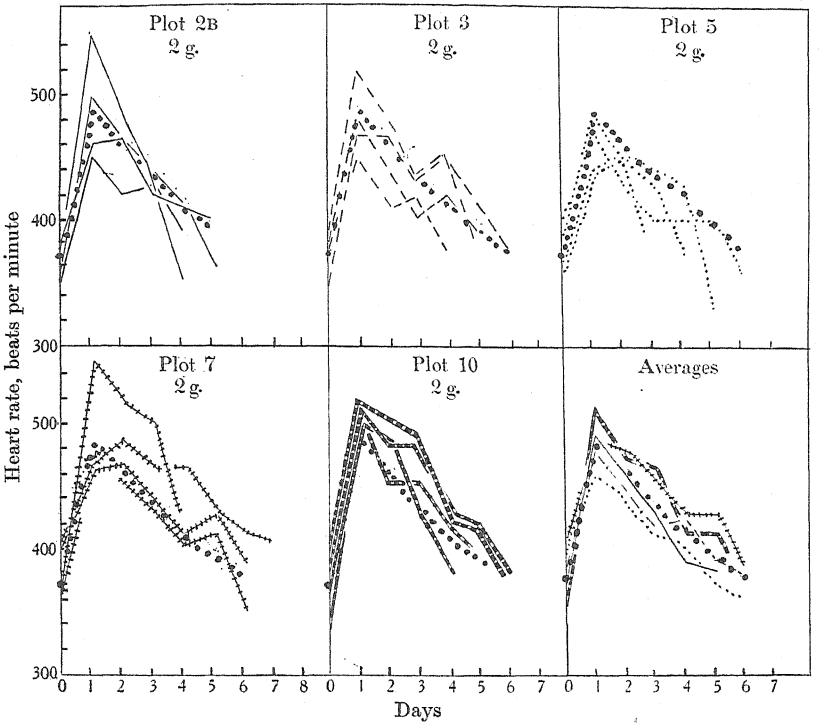

Fig. 8*b*. Comparison of vitamin B_1 activity of whole meal wheat flours, using 4 animals per group. The indication obtained in the preliminary test is confirmed, *viz.* showing the slight superiority of plots 7 and 10 and slight inferiority of plot 5, compared with 2B or 3. *Days protected:* Plots 2B and 3 = 5 days; plot 5 = 4 days; plots 7 and 10 = 6 days. *Relative activities:* Plots 2B and 3 = 100; plot 5 = 80; plots 7 and 10 = 120.

•–•–• = Comparison curve, mean of all specimens. —— = Plot 2B.
– – – = Plot 3. ····· = Plot 5. +++++++ = Plot 7. ▬▬ = Plot 10.

that the vitamin B_1 content when expressed per g. of whole grain is not affected to any important extent (the fuller data will be presented elsewhere). In conformity with this, growth tests on the "sharps" prepared from the grain from these same plots showed no appreciable variation in content of vitamin B complex.

PART VI.

PHYSIOLOGICAL SIGNIFICANCE OF THE BRADYCARDIA IN VITAMIN B_1 DEFICIENCY.

1. *Relations between bradycardia and "polyneuritic" symptoms.*

As mentioned in Part II, the incidence of convulsions in our vitamin B_1-deficient rats was greatly increased when they were given small traces of vitamin B_1 in their diet. This symptom seems to be associated, that is, with hypovitaminosis rather than complete avitaminosis or with chronic rather than acute

vitamin B_1 deficiency. This finding confirms earlier conclusions of Sherman and Sandels [1931] and Sebrell and Elvove [1931]. (A similar relation appears to apply also to certain symptoms of vitamin B_1 deficiency in the pigeon.) Measurements of the heart rate which we have carried out suggest that this small amount of vitamin B_1 probably has the function of keeping the heart rate at a high enough value to enable the animal to survive, thus allowing the chronic symptoms of polyneuritis to develop. We find that the heart rate of a "hypovitaminotic" rat with symptoms of polyneuritic convulsions is generally around 400 beats per minute, while a rat rapidly run out on a diet completely devoid of vitamin B_1 has a much lower heart rate, about 300 beats per minute or lower, and no symptoms of "polyneuritis." In other words the biochemical lesion caused by vitamin B_1 deficiency seems to affect the heart almost immediately and may so cause sudden death from heart failure, while its poisoning action on the central nervous system proceeds more slowly.

2. *Relation to the biochemistry of vitamin B_1 action.*

In the earlier paper on this question [Drury *et al.*, 1930] the suggestion was made that the bradycardia might be correlated with the accumulation of lactic acid which is known to occur in vitamin B deficiency [Bickel, 1924; 1925; Collazo, 1923; Collazo and Morelli, 1926]. This error in lactic acid metabolism seems to be a consistent feature of vitamin B_1 deficiency, is not restricted to any one organ or type of tissue and is common to various species. Work on beri-beri patients in Japan, for example, has shown how the lactic acid tends to reach abnormally high levels, especially after exercise. Hayasaka [1930, see also Hayasaka and Inawashiro, 1928; 1930; Inawashiro and Hayasaka, 1928] has found that the recovery process after muscular exercise is more prolonged in beri-beri than in normal individuals, and, in the case of the dog, he has pointed out that in vitamin B deficiency even quite light exercise sufficed to keep the blood-lactic acid above its normal value for prolonged periods. He therefore concludes that lack of vitamin B leads to a disturbance in the resynthesis of glycogen from lactic acid. In keeping with this view he has found that when sodium lactate was injected into beri-beri patients the resynthesis to glycogen was much lower than in normal controls or cured beri-beri subjects. The accumulation of lactic acid in vitamin B_1 deficiency has been confirmed further by Collazo and Bayo [1931] and others, and Kinnersley and Peters [1930] and their co-workers have recently found a correlation between the imminence of convulsions in pigeons and the excess of lactic acid accumulating in certain parts of the brain. From what is known of the influence of lactic acid on heart rhythm [Clark *et al.*, 1932] it is to be expected that an excess will diminish the rate of beat *in vivo*, as it does in isolated preparations. Measurements of blood-lactic acid (Fig. 9) in vitamin B-deficient rats have confirmed our belief in a correlation between the lactic acid excess and the low heart rate. On the other hand it must be recognised that this does not

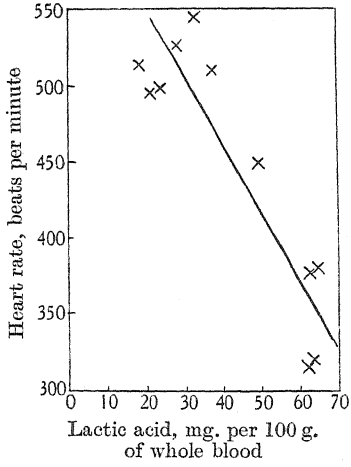

Fig. 9. Correlation between fall in heart rate and rise in lactic acid content of blood in vitamin B_1 deficiency.

prove that the correlation is a direct causative one. Attempts to influence the rate by the administration of large doses of sodium lactate have been unsuccessful.

Experimental. It was found necessary to use about 3 rats in order to obtain enough blood for each lactic acid estimation. The blood samples were obtained by decapitating the rats with scissors over a weighed basin of 20 % trichloroacetic acid. The blood was allowed to drain from the animals for about half a minute, the basin was then again weighed and the amount of blood obtained calculated from the difference in the weighings. The amount of blood used was usually between 6–10 g. this giving a reading of approximately 1–3 ml. of $N/100$ I in the final titration. When sufficient blood had been obtained the trichloroacetic acid mixture was made up to a definite volume so as to give a final concentration of about 5 % trichloroacetic acid. This solution was then filtered and precipitated with the copper-lime reagent to remove carbohydrates and the lactic acid estimation carried out according to the method of Friedemann *et al.* [1927].

As will be seen from Fig. 1 the heart rate of a normal rat is between 500 and 600 beats per minute, and the lactic acid content is about 30 mg. per 100 g. of blood. When the rats become deficient in vitamin B_1 the heart rate drops to 300–350 beats per minute and the lactic acid content rises to about 70 mg. per 100 g.

3. *Vitamin B_1 as oxidative coenzyme.*

The existing evidence warrants the suggestion that the physiological rôle of vitamin B_1 is to intervene at some stage in that complex cycle of reactions in carbohydrate metabolism involving the formation and oxidation of lactic acid, and in a capacity corresponding with that of coenzyme. Banga and Szent-Györgyi [1932] announced the isolation of a coenzyme for lactic acid dehydrogenase. One of us has drawn attention elsewhere [Harris, 1934] to the fact that a number of salient properties of the coenzyme bear a resemblance, superficially at least, to those of vitamin B_1—*e.g.* it is needed for the normal functioning of the heart muscle (it was from this source that it was isolated), and it is concerned in the removal and oxidation of lactic acid. In this connection also its suggested relationship to adenine, or adenylic acid, which is known to be of significance for the physiology of the heart beat, is of further interest.

It seems certain of course that vitamin B_1 itself cannot be identical with Banga and Szent-Györgyi's coenzyme (*cf.* Boyland, 1933); nevertheless there appears to be a close interconnection between the two classes of substances and their physiological rôles in the related and interdependent series of reactions which they control. The nature of the lactic acid dehydrogenase system is discussed further in the following paper [Birch and Mann, 1934].

Summary.

Part I.

The technique of estimating vitamin B_1 by the heart rate method which has been in use in this laboratory for several years as a standard method is described. Single doses of the unknown are fed to rats suffering from vitamin B_1 deficiency and the heart rate measured at daily intervals for a few days afterwards. The magnitude and duration of the effect are proportional to the amount of vitamin so given. The accuracy of the method was checked by tests on four substances chosen for investigation by the Sub-committee on Vitamin B_1 Standardisation, *viz.* activated acid clay standard, dried yeast, marmite and wheat germ. These were assayed by separate control tests by the independent methods described in Parts II and III.

Part II.

A method of carrying out vitamin B_1 determinations by curative tests on polyneuritic convulsions in rats is described. A feature of this method is the close agreement in response between animals receiving duplicate doses.

Part III.

Results with a growth rate method are also given and the conditions defined for the most accurate results. The number of variables differs too much to permit the use of a standard dose-effect reference curve—as for example with the amount of autoclaved marmite given as the source of vitamin B_2. Under the conditions described differences of ± 20 % in activity are readily detected, and 3 out of 5 animals at least in a group fall within ± 15 % of the median value while the values reached in the assays by any of the three rat methods appeared to vary within an experimental error of about ± 5 %.

Part IV.

Results by the heart rate method correspond with those given by the other methods, the comparative values found, taking activated clay standard as 100, being yeast 18·8, 19·3, 20, 18; marmite 8·3, 8·7, 9·1, 8·5; wheat germ 7, 6·9, 7·2, by the bradycardia, convulsions, growth rate and pigeon (head retraction) methods respectively. Among the advantages of the heart rate method are its convenience, rapidity, simplicity and economy, and the fact that it can be readily used for determining foodstuffs containing only small amounts of vitamin B_1 which are beyond the scope of the curative pigeon or rat (convulsions) method, and—when they contain starch and give rise to refection—the rat growth test.

Part V.

As an example of the use of the heart rate method tests are described on white and brown breads and other wheat products. These could not be readily estimated by the pigeon or rat curative methods, and growth tests on rats were vitiated by the occurrence of refection.

Part VI.

A rat given no vitamin B_1 and dying from acute avitaminosis has a very low heart rate (*e.g.* 300 per min.) and no polyneuritic convulsions. A rat given slight traces of vitamin B_1 and developing chronic hypovitaminosis has a less severe bradycardia (*e.g.* heart rate 400) but convulsions. It is suggested that the traces of vitamin B_1 act by keeping the heart rate at a high enough level to enable the animal to survive and so permit the more slowly developing chronic symptom to develop.

The earlier suggestion is confirmed that the bradycardia is correlated with the accumulation of lactic acid in the vitamin B_1-deficient animal. Vitamin B_1 appears to have the property of a coenzyme-like substance intervening in the chain of carbohydrate oxidation reactions.

REFERENCES.

Banga and Szent-Györgyi (1932). *Biochem. Z.* **246**, 203.
Bickel (1924). *Biochem. Z.* **146**, 493.
—— (1925). *Biochem. Z.* **166**, 251.
Birch and Mann (1934). *Biochem. J.* **28**, 622.
Boyland (1933). *Biochem. J.* **27**, 786.
Clark, Gaddie and Stewart (1932). *J. Physiol.* **75**, 331.
Collazo (1923). *Biochem. Z.* **136**, 278.
—— and Bayo (1931). *Biochem. Z.* **238**, 335.
—— and Morelli (1926). *J. Physiol. Path. Gén.* **24**, 76.
Coward, Burn, Ling and Morgan (1933). *Biochem. J.* **27**, 1719.
Drury, Harris and Maudsley (1930). *Biochem. J.* **24**, 1632.
Friedemann, Cotonio and Schaffer (1927). *J. Biol. Chem.* **73**, 335.
Harris (1934). *Ann. Review Biochem.* **3**.
Hartwell (1924). *Biochem. J.* **18**, 120, 1323.
Hayasaka (1930). *Tôhoku J. Exp. Med.* **14**, 72, 85, 283, 487.
—— and Inawashiro (1928). *Tôhoku J. Exp. Med.* **12**, 29.
—— —— (1930). *Tôhoku J. Exp. Med.* **14**, 53.
Inawashiro and Hayasaka (1928). *Tôhoku J. Exp. Med.* **12**, 1.
Kinnersley and Peters (1930). *Biochem. J.* **24**, 711.
McCarrison and Viswanath (1926). *Ind. J. Med. Res.* **14**, 351.
Mottram and Hartwell (1929). *Lancet*, ii, 892.
Rowlands and Wilkinson (1930). *Biochem. J.* **24**, 199.
Sebrell and Elvove (1931). *U.S. Pub. Health Reps.* **46**, 917.
Sherman and Sandels (1931). *J. Nutrition*, **3**, 395.
Smith (1930). *U.S. Pub. Health Reps.* **24**, 116.

LXXXVI. THE ACTIVATION OF LACTIC DEHYDROGENASE AND ITS RELATION TO THE RÔLE OF VITAMIN B_1.

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THE necessity of a coenzyme for the oxidation of lactic acid by the lactic dehydrogenase of animal tissues has been shown by Szent-Györgyi [1925]. The preparation of a concentrate of this substance from mammalian heart tissue has been described by Szent-Györgyi and Vargha [1932]. Recently Banga and Szent-Györgyi [1933] have further purified the concentrate by precipitation with phosphotungstic acid and picric acid and have obtained the coenzyme as a semi-crystalline picrate. By investigation of the products of hydrolysis of the coenzyme they concluded that it was a monophosphate of a monoadenylnucleotide not identical with muscle adenylic acid or adenylpyrophosphate, but possibly identical with cozymase.

This coenzyme is of considerable interest owing to the importance of lactic acid as an intermediary in animal metabolism. In certain pathological conditions where accumulation of lactic acid is observed, it appears possible that such an accumulation may be due to a deficiency of the coenzyme. Such an accumulation occurs as a symptom of vitamin B_1 deficiency and in this case there is additional evidence which suggests the possibility of a connection between vitamin B_1 activity and the coenzyme activity.

Kinnersley and Peters [1929; 1930] showed that brain tissue of polyneuritic pigeons had an abnormally high lactic acid content. Fisher [1931] has shown that there is a general defect in lactic acid metabolism in the polyneuritic pigeon as a whole, in addition to the defect in brain described by Kinnersley and Peters. This defect in lactic acid metabolism in vitamin B_1 deficiency is not confined to pigeons. According to Japanese workers, *e.g.* Hayasaka [1930], lactic acid accumulates in the body and reaches abnormally high levels in patients suffering from beri-beri.

Further, a relation between vitamin B_1 and lactic dehydrogenase coenzyme is suggested from the work of Meiklejohn *et al.* [1932] and Passmore *et al.* [1933], who showed that the cerebrum of the brain of polyneuritic pigeons when minced and mixed with lactic acid showed a low oxygen uptake compared with normal brain tissue. The addition of vitamin B_1 concentrate increased the *in vitro* respiration of the polyneuritic brain tissue, and it was assumed that the oxidation affected in this way was that of lactic acid.

Unfortunately, from the recent work of Meiklejohn [1933] it appears that the amount of lactic acid removed during the oxidation is not influenced by the presence of the vitamin. A similar observation has been made by Hahn and Fischbach [1929] using ox muscle. They find that with washed ox muscle, the

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renewed oxygen uptake in presence of "Kochsaft" is not due to oxidation of the added lactic acid, for although the amount of respiration may be restored to 90 % of its original value by adding the "Kochsaft," the lactic acid content remains unaltered. On the other hand, while avitaminous brain tissue shows a low oxygen uptake with pyruvic acid similar to that with lactic acid, according to the conclusions of Meiklejohn *et al.* [1933] in this case the addition of vitamin B_1 is without effect. However, on consideration of the results of Peters and his collaborators it appears that this conclusion is somewhat unjustified. The difference in the rate of oxygen uptake between normal and avitaminous brain tissue in presence of lactate is small. Hence the increase obtained on addition of vitamin B_1 to the avitaminous brain is almost sufficient to raise the rate of oxygen uptake to that of normal brain tissue + lactate. On the other hand the difference in the rate of oxygen uptake in the case of normal and avitaminous brain with pyruvate is much greater than in the case of lactate. Hence in this case while addition of vitamin B_1 causes an increase of the same order as in the case of lactate, this addition is insufficient to raise the rate to normal. In the case of lactate and avitaminous brain, using the cerebrum the increase in oxygen uptake produced by addition of vitamin B_1 is 28 %, using the optic lobes 22 %. In the case of pyruvate with the cerebrum the increase in oxygen uptake produced by addition of vitamin B_1 is 20 %, and with the optic lobes 40 %. If the increase in the presence of lactate is to be regarded as significant it appears that the case of pyruvate should not be ignored merely because it does not raise the rate of oxygen uptake to normal. Furthermore since the difference in the rate of oxygen uptake between normal and avitaminous brain with pyruvate is greater than with lactate, it appears that deficiency of vitamin B_1 produces actually a greater effect on the mechanism of pyruvate oxidation than on that of lactate oxidation. Peters explains his conclusion by assuming that the increase obtained with pyruvate is due to the effect of the vitamin on the residual lactic present in the brain tissue.

The question of the possible identity of vitamin B_1 and lactic dehydrogenase coenzyme has been investigated by Boyland [1933]. As vitamin B_1 is a sulphur-containing compound and the coenzyme contains no sulphur, it is difficult to imagine how the two can be identical. Further arguments point in the same direction, for example Szent-Györgyi precipitates his coenzyme with mercuric nitrate, followed by precipitation from aqueous solution with acetone. By neither of these reagents would vitamin B_1 be precipitated. Of course it still remains possible that vitamin B_1 might exist in the coenzyme in some such form as the base of a nucleotide which occurred as a small impurity in Szent-Györgyi's preparation, but Boyland's experiments do not allow for this.

Boyland concluded that crude coenzyme preparations possessed only a slight vitamin B_1 activity as determined by rat feeding experiments, and that while crude vitamin B_1 concentrates showed activity as coenzyme for heart-lactic dehydrogenase, purified preparations did not possess this activity.

Table I shows the results obtained by Boyland in testing the activity of his coenzyme preparation by the methylene blue technique.

Table I.

Lactic dehydrogenase coenzyme solution 10 mg. per ml.	*M*/10 lactate ml.	Reduction time in minutes
0·0	1·0	180
0·1	1·0	47
0·2	1·0	27
0·4	1·0	12½
0·4	0·0	150

Since in the above experiments doubling the concentration of the coenzyme doubles the velocity of the reaction, it appears doubtful whether 0·4 ml. of the coenzyme solution is sufficient to produce maximum activation of the enzyme. Meiklejohn *et al.* [1932] found in their experiments with avitaminous brain tissue and lactate that it was necessary to add only 1/10 of a pigeon dose of vitamin B_1 to each Barcroft cup to obtain the maximum increase in oxygen uptake. Thus in the case of Boyland's results, if it is assumed that 0·4 ml. of the coenzyme solution produces maximum activation of the enzyme then this amount should be equivalent to 1/10 of a pigeon dose. Boyland tested the vitamin B_1 activity of his preparation by rat feeding experiments. He found, as would be expected, that 0·1 ml. and 0·2 ml. showed no activity, while 2 ml. showed some activity, but less than one pigeon dose. From the above calculation, 2 ml. should only be equivalent to half a pigeon dose or less.

Thus Boyland's conclusion that lactic dehydrogenase coenzyme possesses little vitamin B_1 activity appears unjustified and in spite of his work it still seems possible that there may be a close interrelation between vitamin B_1 and the coenzyme activity. The work to be described was started with the view of investigating this possibility.

Preparation of the enzyme.

During the course of this work three different methods have been employed for the preparation of lactic dehydrogenase from heart muscle. The methods so employed may be divided into two sections according to the properties of the enzyme preparation obtained.

Section I. Szent-Györgyi and Vargha's method [1932]. 40 g. pig heart muscle freed from fat and connective tissue are finely chopped and shaken with 800 ml. of distilled water at room temperature for 10 minutes; after squeezing through linen the residue is shaken with another 800 ml. of distilled water for a further 10 minutes. After squeezing through linen, the residue is placed in a mortar and frozen by surrounding it with an ice and salt freezing mixture. The tissue is then ground up and mixed with 120 ml. of $M/15$ phosphate buffer at p_H 7·2.

Section II. (*a*) *Boyland's method* [1933]. 20 g. of well minced pig's heart are ground up with 250 ml. of ice-cold water and pressed through linen. The residue is washed in this way six times, pressed and then frozen. While frozen it is minced three times to break up the cells and then suspended in 100 ml. $M/100$ phosphate buffer at p_H 7·4.

(*b*) *Birch and Mann's method.* In this method the enzyme is extracted from the tissue. The method is identical in its initial stages with that of Szent-Györgyi. It is essential that the frozen tissue should be thoroughly ground up so as to break up the cells as far as possible. The pounded tissue is suspended in phosphate buffer and allowed to stand for 2–3 hours at room temperature. The suspension is then squeezed through linen, and the liquid obtained is fully saturated with ammonium sulphate. The precipitate is filtered off and dried in a vacuum-desiccator. The yield from 100 g. of tissue is about 7 g. This enzyme preparation shows little loss of activity after several weeks if it is left in the dry form in a vacuum-desiccator. It may be further purified by dissolving in phosphate buffer, centrifuging off the insoluble residue and then reprecipitating with ammonium sulphate.

The differentiation of the methods of preparation is based on the amount of washing which the tissue undergoes. Szent-Györgyi's enzyme preparation consists of comparatively unwashed tissue, while by Boyland's method the tissue is

very thoroughly washed. In our own method the enzyme is further purified by extraction from the tissue and by precipitation with ammonium sulphate. This difference in the degree of washing of the tissue is accompanied by a fundamental difference in the properties of the resultant enzyme preparations.

Preparation of the coenzyme.

At the beginning of this work the method of Szent-Györgyi and Vargha [1932] was used for the preparation of the coenzyme from bullock's heart and Boyland's enzyme was used as a means of testing the activity of the coenzyme. The yield of the coenzyme is small—about 0·7 g. of the crude concentrate from 5 kg. of heart, and in the subsequent purification by phosphotungstic acid most of the activity was lost. In place of the phosphotungstic precipitation other methods were used for the purification of the crude coenzyme, and in some cases it was apparently separated into two fractions, either of which separately showed little coenzyme activity but together gave good activation of the enzyme. An example of this, where precipitation with silver nitrate was used as the means of fractionation is shown in Table II.

Solution I. The crude coenzyme solution is precipitated at p_H 1·5 with $AgNO_3$. The precipitate is decomposed with H_2S, evaporated to a small volume and precipitated by the addition of 10 volumes of acetone. This precipitate dissolved in water forms solution I.

Solution II. The filtrate from the above acid silver precipitate is brought to p_H 6 with ammonia and again precipitated with $AgNO_3$. The filtrate is treated with H_2S, evaporated and precipitated with acetone. The acetone filtrate is evaporated until all the acetone is removed.

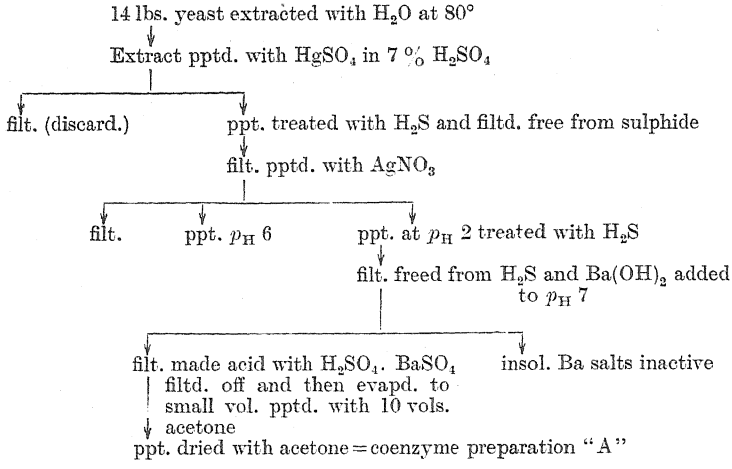
Using these two solutions with Boyland's enzyme the following results were obtained by the Thunberg technique of methylene blue reduction. The total volume in each tube was 3 ml. In general 1 ml. of enzyme was used and 1 ml. of 1 in 5000 methylene blue, together with phosphate buffer at p_H 7·4. In some cases when the enzyme was found to be weak the methylene blue was cut down to 0·5 or 0·25 ml.

Table II.

	Reduction time (mins.)
0·25 ml. of (I) + enzyme + lactate + MB	80
1·0 ml. of (II) + enzyme + lactate + MB	120
0·25 ml. of (I) + 1·0 ml. of (II) + enzyme + lactate + MB	10
0·5 ml. of (I) + 0·5 ml. of (II) + enzyme + MB (no lactate)	68

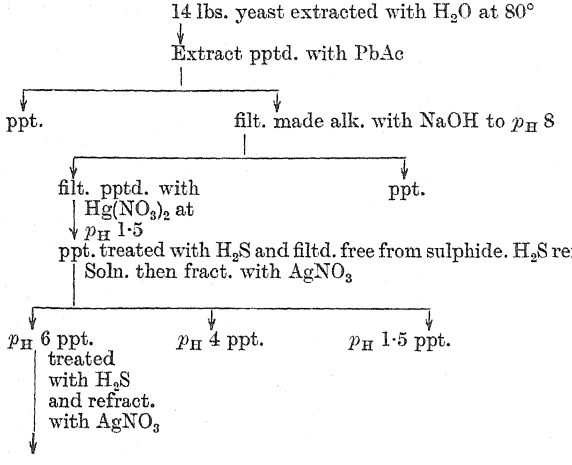
Attempts to separate these two factors from heart completely were unsuccessful, though results such as those shown in the above table were sometimes obtained. One of the causes of this failure was the minute amounts of the substances with which we were dealing. It was found that yeast contained considerable amounts of the coenzyme. Since yeast is more readily obtainable and easier to work up on a large scale than heart tissue, it formed the source of most of our subsequent preparations of coenzyme. Even using yeast it has so far proved impossible to effect a complete separation of the two factors. One factor, which is probably identical with Szent-Györgyi's coenzyme is readily obtained more or less free from the other. The method of obtaining this is shown in the following scheme.

Preparation A.



Attempts to obtain the second factor free from the first have up to the present been unsuccessful. The best preparations so far consist of a mixture of the two factors. The method of preparation of this mixture is shown in the following scheme.

Preparation B.



Most of the activity is found in the silver ppt. at p_H 6 while some activity is also found in the p_H 1·5 ppt. These ppts. are again fractionated with silver nitrate. The silver fractionation does not give clear cut results. The majority of the activity usually comes down at p_H 6, but often some comes down at p_H 1·5. These solutions obtained after refractionation with silver are called "coenzyme preparation B" in the subsequent experiments.

Using preparation "A" in the Thunberg technique of methylene blue reduction only small amounts are necessary to produce maximum activation (0·5 to 2 mg.; the actual amount seems to vary with the enzyme used); the activation produced is small. With preparation "B" relatively large amounts are often necessary but the activation produced is 5–8 times as great when using

the pure enzyme. It is seen from the schemes of preparation shown that the first factor "A" is precipitated by $HgSO_4$ and by acetone from aqueous solution. The second factor does not seem to follow these reactions, but if the $HgSO_4$ filtrate or the acetone filtrate is examined for coenzyme activity it is usually found to possess only slight activity. In some cases by mixing the two solutions reactivation has been obtained as is shown in Table II, but more often than not the mixture is found to be inactive. Thus it appears that the second factor is unstable except when in the presence of the first, and the best preparation obtainable is a mixture of the two, containing a greater percentage of the second factor.

Further evidence for the existence of two factors.

In addition to the results shown in Table I other evidence has been found which suggests that two factors are necessary. This has been obtained by comparing the maximum activation produced by coenzyme preparations "A" and "B" with each type of enzyme.

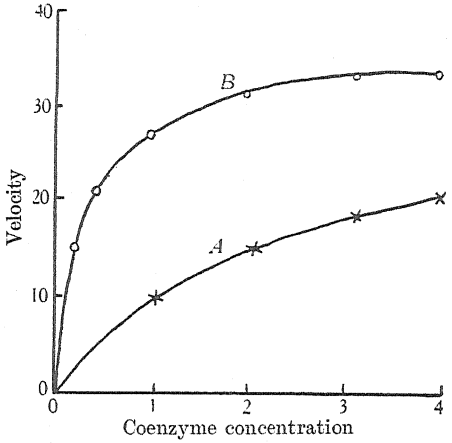


Fig. 1.

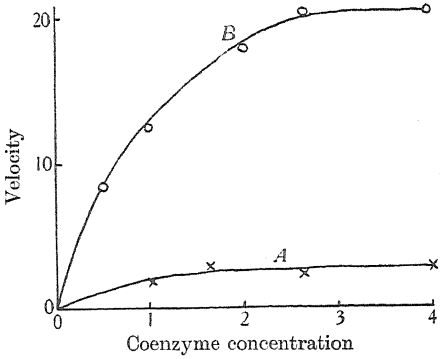


Fig. 2.

Fig. 1. Comparison of the two coenzyme preparations "A" and "B" using the unpurified enzyme at p_H 7·2. Abscissa = relative amounts of coenzyme added; ordinate = $1/T$.

In this experiment 1 ml. of Szent-Györgyi's enzyme was used with 1 ml. of 1 in 5000 methylene blue, the lactate concentration being $M/10$. The minimum reduction time with coenzyme preparation "B" was 3 minutes. The minimum reduction time with coenzyme preparation "A" was 5 minutes. The blanks were enzyme + lactate + MB = 135 minutes, enzyme + maximum amount of coenzyme "B" + MB = 43 minutes, enzyme + coenzyme "A" + MB = 150 minutes.

Fig. 2. Comparison of coenzyme preparations "A" and "B" with the purified enzyme at p_H 7·2. This enzyme was twice precipitated with ammonium sulphate. Starting with 1 g. of the original precipitate the final volume was made up to 20 ml., and ½ ml. of the solution was used. The minimum reduction time with coenzyme preparation "B" was 4¾ minutes. The minimum with coenzyme preparation "A" 31½ minutes. The blanks were enzyme + lactate + MB = 6 hours + ; enzyme + coenzyme "A" + MB = 6 hours; enzyme + coenzyme "B" + MB = 51 minutes.

Fig. 1 shows curves obtained using Szent-Györgyi's enzyme; curve A with increasing amounts of coenzyme "A," curve B with coenzyme "B." With this unpurified enzyme coenzyme "A" gave an activation which was almost two-thirds of that obtained with "B."

When a more purified enzyme was used with increasing amounts of the coenzyme results were obtained which are shown in Fig. 2. Here it is seen that

maximum activation with coenzyme "A" was only one-seventh of that given by coenzyme "B."

Thus using an enzyme consisting of practically unwashed tissue both coenzymes show similar activity. Using a purified enzyme there is a marked difference in the activation produced by the coenzyme preparations "A" and

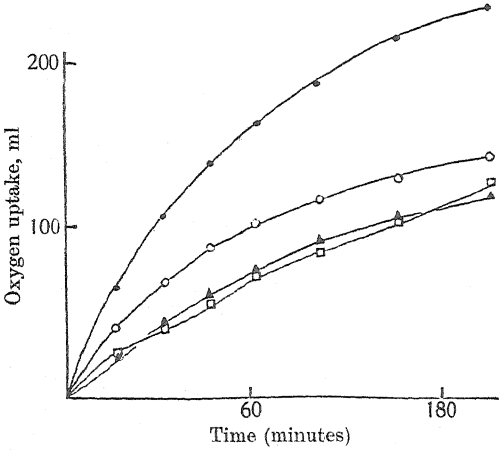


Fig. 3.

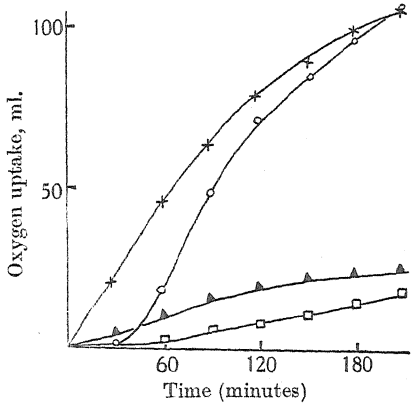


Fig. 4.

Fig. 3. Rate of oxygen uptake using Szent-Györgyi's enzyme at p_H 7·2.

▲, {Right-hand cup contained enzyme + lactate + coenzyme "A"
{Left-hand cup contained enzyme + lactate

□, {Right-hand cup contained enzyme + lactate + coenzyme "A"
{Left-hand cup contained enzyme + coenzyme "A"

●, {Right-hand cup contained enzyme + lactate + coenzyme "B"
{Left-hand cup contained enzyme + lactate

○, {Right-hand cup contained enzyme + lactate + coenzyme "B"
{Left-hand cup contained enzyme + coenzyme "B"

2 ml. of enzyme were used, the lactate concentration being $M/20$. The amount of coenzyme used was that which was found necessary to give maximum activation in 3 ml. when using the methylene blue technique.

The difference between the two curves obtained with coenzyme "B" is presumably due to some blank in the coenzyme preparation, which is accounted for in ○ where it is balanced against enzyme + coenzyme.

Fig. 4. Oxygen uptake using ammonium sulphate enzyme at p_H 7·2.

×, {Right-hand cup contained enzyme + lactate + coenzyme preparation "B"
{Left-hand cup contained enzyme + lactate

○, {Right-hand cup contained enzyme + lactate + coenzyme preparation "B"
{Left-hand cup contained enzyme + coenzyme preparation "B"

▲, {Right-hand cup contained enzyme + lactate + coenzyme preparation "A"
{Left-hand cup contained enzyme + lactate

□, {Right-hand cup contained enzyme + lactate + coenzyme preparation "A"
{Left-hand cup contained enzyme + coenzyme preparation "A"

Ammonium sulphate coenzyme 1 g. made up to 18 ml. in phosphate buffer, then 2 ml. used. The lactate and coenzyme concentrations were the same as in the previous experiment.

"B." With a purified enzyme preparation "A" is practically inactive. Thus preparation "B" contains an additional factor necessary for the activation of lactic dehydrogenase. This factor is present in Szent-Györgyi's crude enzyme, and thus coenzyme preparation "A" shows activity. On purification of the enzyme

however this factor is removed and preparation "A" becomes inactive with this enzyme, while preparation "B" still remains active.

This explanation is again supported by similar results obtained by using the Barcroft technique for the measurement of the rate of oxygen uptake. The results using Szent-Györgyi's enzyme are shown in Fig. 3. The amount of coenzyme added was the same as that which was required to give maximum activation in 3 ml. when using the Thunberg technique. It is seen that coenzyme "A" gives a rate of oxygen uptake which is about half that given by using coenzyme "B."

When using the purified enzyme curves were obtained which are shown in Fig. 4. In this experiment the rate of oxygen uptake with coenzyme "A" is only one-fifth of the rate obtained by using coenzyme "B."

Thus the results obtained by the Barcroft technique support those obtained by the Thunberg technique and indicate that two factors are necessary for the activation of lactic dehydrogenase. In the Barcroft experiment just described with the ammonium sulphate enzyme the partially purified enzyme was used, not that purified by a second precipitation with $(NH_4)_2SO_4$. This was because by further purification of the enzyme in this manner we appear to remove a factor necessary for oxygen uptake by the system. This is shown in Fig. 5 where we have used the twice precipitated enzyme with coenzyme preparation "B" with and without methylene blue.

It is seen that the addition of methylene blue enables the system to take up oxygen about twice as fast as when methylene blue is absent. The system has not yet been obtained in such a state that it gave no oxygen uptake without methylene blue but these results seem to indicate that lactic dehydrogenase with its coenzymes is unable to take up oxygen by itself. Apparently it needs some system such as the cytochrome system to enable it to take up oxygen. The addition of methylene blue to systems containing the cruder enzyme has no effect on the oxygen uptake.

The possible identity of these coenzymes with cozymase.

In his latest paper Szent-Györgyi suggests that his factor is possibly identical with cozymase. During the course of this work two preparations of cozymase have been made according to the method of Euler and Myrbach [1928] and it was found that both were completely inactive as coenzyme for lactic dehydrogenase, while they showed a high activity as coenzyme for glucose dehydrogenase [Mann, 1932]. The estimation of the preparations as cozymase is at present being carried out and until this is done it cannot be claimed definitely that cozymase is inactive as the coenzyme. But from the results with one cozymase preparation which are shown in Table III and from the fact that the method of preparation of cozymase differs markedly from the preparation of lactic coenzyme the conclusion that they are not identical is fairly safe.

Table III. *Activity of cozymase as lactic coenzyme. Cozymase prepared according to Euler and Myrbach [1928]. Szent-Györgyi's crude enzyme used.*

	R.T.
0·1 mg. cozymase + enzyme + lactate + MB	4 hrs. +
0·5 mg. ,,	4 hrs. +
2·5 mg. ,,	4 hrs.
4·0 mg. ,,	4 hrs.
Blank with lactate + enzyme + MB	4 hrs. +
1·6 mg. P.T.A. heart coenzyme + enzyme + lactate + MB	4¼ mins.

Inhibition by pyruvic acid.

It is difficult to understand why for a simple reaction such as the conversion of lactic acid into pyruvic acid two coenzymes should be necessary. Is the second factor a coenzyme of this reaction or of some subsequent one? Using the Thunberg technique with purified enzyme and coenzyme preparation "A" or, as we

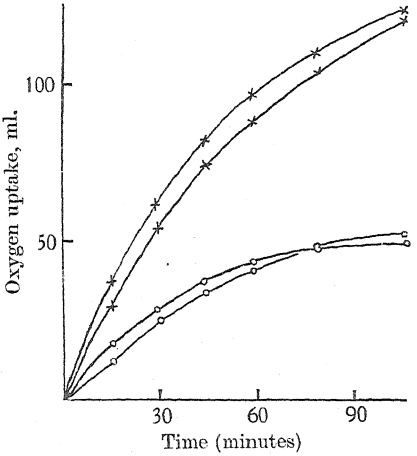


Fig. 5.

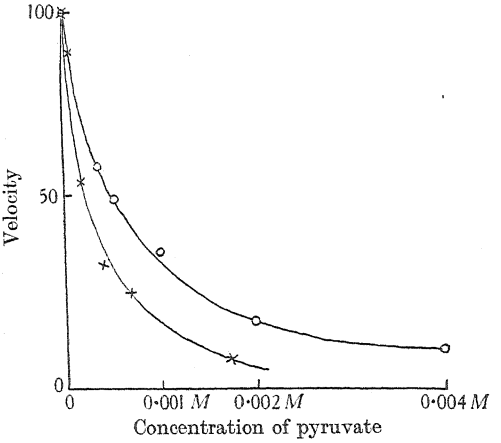


Fig. 6.

Fig. 5. The difference between the rate of oxygen uptake when methylene blue is added to the purified lactic dehydrogenase system.

o, { Right-hand cup contained enzyme + lactate + coenzyme preparation "B"
Left-hand cup contained 3 ml. phosphate buffer.

×, { Right-hand cup contained enzyme + lactate + coenzyme preparation "B" + MB
Left-hand cup contained 3 ml. phosphate buffer.

The enzyme was prepared by dissolving the 1st ammonium sulphate precipitate in phosphate buffer, centrifuging off the insoluble matter and then reprecipitating with ammonium sulphate. 0·5 g. of this precipitate was made up to 8 ml. and 1 ml. was used in each cup.

Fig. 6. Inhibition of the activity of lactic dehydrogenase by small amounts of pyruvic acid.

o, Coenzyme preparation "B"
×, Coenzyme preparation "A"

Ammonium sulphate-precipitated enzyme used: 1 g. made up to 28 ml. 0·5 ml. of this solution was used with coenzyme preparation "B." The minimum reduction time (*i.e.* without pyruvate) was $2\frac{3}{4}$ minutes and the maximum when pyruvate was added 28 minutes. The blank on coenzyme + enzyme alone was 68 minutes. 1 ml. of the enzyme solution was used with coenzyme preparation "A" and a minimum reduction time of $6\frac{3}{4}$ minutes was obtained. The maximum when pyruvate was added was 98 minutes.

The concentration of lactate was $M/10$, and 1 ml. of 1 in 5000 methylene blue was used throughout. The pyruvic acid was purified according to the method of Case [1932], and was added as the sodium salt.

believe, Szent-Györgyi's coenzyme, it was often found that for a short period reduction of methylene blue proceeded quite rapidly, after which further reduction was very slow. It therefore appeared that some substance which was being formed was inhibiting the reaction and it appeared probable that this substance might be pyruvic acid. This view was supported by results obtained from the effect of added pyruvic acid on the velocity of reduction of methylene blue in presence of $M/10$ lactate. The results are shown in Fig. 6.

It is seen from Fig. 6 that a minute concentration of pyruvate inhibited the reaction, using coenzyme "A" with the ammonium sulphate enzyme. This may

afford an explanation of the slowing off in the speed of reduction of M.B. when this coenzyme was used, as immediately a trace of pyruvic acid was formed it inhibited the reaction.

Using coenzyme "B" a much greater concentration of pyruvate was necessary to give the same percentage inhibition. It is suggested as an explanation of this that in the case of coenzyme "B" some factor was present which enabled the removal of pyruvic acid. In the case of coenzyme "A" this factor was missing.

The second factor present in the coenzyme "B" would therefore be a co-enzyme for removal of pyruvic acid.

One would naturally suppose that this removal would be carried out by oxidation, but if this is so it seems that the oxidation is not accomplished by pyruvic dehydrogenase since pyruvate without lactate in the presence of enzyme and coenzyme hardly reduced methylene blue at all.

Fig. 6 shows that an apparent equilibrium or maximum inhibition with pyruvate was obtained with a pyruvate concentration between 0·002 and 0·004 *M*. This differs from the results of Bernheim [1928] who found that this "apparent equilibrium" was obtained between 0·033 and 0·066 *M* concentration of pyruvate. This was presumably due to the presence of our second factor in his enzyme. It must be borne in mind however that it has not yet been shown that the soluble lactic dehydrogenase from yeast needs a coenzyme but, as the co-enzyme for heart muscle-lactic dehydrogenase occurs in relatively large amounts in yeast, it is conceivable that it does. This matter is at present under investigation.

Activity of the coenzyme preparations "A" and "B" as vitamin B_1.

The difference between the coenzymes "A" and "B" as activators of lactic dehydrogenase is accompanied by a difference in their action on vitamin B_1-deficient rats. Rats as the experimental animals have been used and the heart rate method as the method of estimating vitamin B_1 activity. Normal rats (heart rate 500–550); vitamin B_1-deficient rats (heart rate 300–350). Injection of vitamin B_1 intraperitoneally causes the heart rate of vitamin B_1-deficient rats to return to normal, usually in about 6 hours.

Injection of coenzyme "A" into vitamin B_1-deficient rats caused an almost instantaneous drop in heart rate. This supports Szent-Györgyi's opinion that his coenzyme is an adenylic acid, as Drury and Szent-Györgyi [1929] have shown that certain adenine compounds, notably adenosine and adenylic acid, caused bradycardia in normal rats when injected. Using coenzyme "A" this drop in the heart rate lasted for 1–2 hours depending on the amount injected and then the rate returned to nearly its original value. It showed no rise above its original value and therefore no vitamin B activity.

In these experiments that amount of coenzyme which produced maximum activation in a total volume of 3 ml. in a M.B. tube was called one dose.

Using the coenzyme "B," if 9 doses were injected a similar drop in heart rate was produced to that obtained with coenzyme "A," showing that the adenine compound was again present. The heart rate returned to its original value after 1–2 hours, and tended to rise slightly above the original value, but this slight rise was only maintained for a few hours. Using half the amount the drop was less, and the subsequent rise was quicker, the heart rate returning to its original value in about half an hour and continuing to rise above its original value during the next 2 or 3 hours. This seemed to show some definite curative effect.

Using $2\frac{1}{2}$ doses, while the initial drop in heart rate was smaller and recovery to original rate more rapid, the subsequent rise in heart rate was much smaller than with $4\frac{1}{2}$ doses. These results are shown in Fig. 7.

The curative effect of the coenzyme "B" was limited to a few hours whereas injection of vitamin B_1 with or without the coenzyme gave a curative effect lasting for several days.

This was shown in Fig. 8 where 9 doses of coenzyme "B" and vitamin B_1 were injected simultaneously.

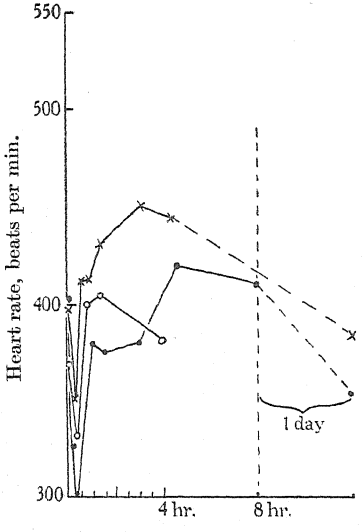


Fig. 7.

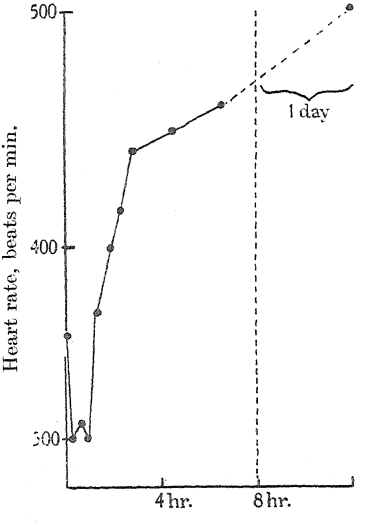


Fig. 8.

Fig. 7. Effect of coenzyme preparation "B" on the heart rate of vitamin B_1-deficient rats. One dose is the amount of coenzyme necessary to give maximum activation of the enzyme in 3 ml.

•, 9 doses injected intraperitoneally
×, $4\frac{1}{2}$ doses injected intraperitoneally
⊙, $2\frac{1}{4}$ doses injected intraperitoneally

The coenzyme preparation used in this experiment was prepared by a slight modification of scheme B. The original yeast extract was first of all treated with acid clay at p_H 4·5 in order to adsorb as much vitamin B_1 as possible. The acid clay was then filtered off and the filtrate treated according to scheme B. The coenzyme is apparently not adsorbed by the acid clay.

Fig. 8. Injection of 9 doses of coenzyme preparation "B" + vitamin B_1 concentrate. Approximately two international units of vitamin B_1 were used.

It seems from this evidence therefore that if vitamin B_1 occurred in the coenzyme it only occurred in a minute amount, and it appears doubtful whether the rise in heart rate produced was due to vitamin B_1. It is therefore unlikely that vitamin B_1 or any compound containing it participates in the oxidation of lactic acid by the systems described in this paper. Nevertheless from Peters's evidence, and from the fact that a temporary cure of the bradycardia is produced by coenzyme "B" it is possible that it is this second factor, which is missing in vitamin B_1 deficiency; but it is obviously useless to form any rigid theory until more evidence has been obtained.

Summary.

A soluble cell-free lactic dehydrogenase from heart muscle has been prepared in a less impure form than hitherto obtained. By using this enzyme it has been shown that two factors are necessary for its activation. Methods for the preparation from yeast of the first factor, which seems to be identical with Szent-

Györgyi's coenzyme, and for a mixture of the two factors have been worked out. Neither of the factors appeared to be identical with cozymase.

Using the system enzyme + lactate + mixture of two coenzymes, addition of methylene blue was found to be necessary before the system could take up oxygen.

Some evidence has been obtained which tends to show that the second coenzyme may be concerned with the removal of pyruvic acid.

The coenzymes have been obtained free from vitamin B_1 but the second factor seems to show some temporary curative effect on the bradycardia produced in vitamin B_1-deficient rats.

We wish to express our thanks to Prof. Sir F. G. Hopkins and Dr L. J. Harris for the interest they have shown in this work.

REFERENCES.

Banga and Szent-Györgyi (1933). *Z. physiol. Chem.* **210**, 228.
Bernheim (1928). *Biochem. J.* **22**, 1178.
Boyland (1933). *Biochem. J.* **27**, 786.
Case (1932). *Biochem. J.* **26**, 753.
Drury and Szent-Györgyi (1929). *J. Physiol.* **68**, 213.
Euler and Myrbach (1928). *Z. physiol. Chem.* **177**, 237.
Fisher (1931). *Biochem. J.* **25**, 1410.
Hahn and Fischbach (1929). *Z. Biol.* **89**, 149.
Hayasaka (1930). *Tôhoku J. Exp. Med.* **14**, 85, 283, 487.
Kinnersley and Peters (1929). *Biochem. J.* **23**, 1126.
—— —— (1930). *Biochem. J.* **24**, 710.
Mann (1932). *Biochem. J.* **26**, 785.
Meiklejohn (1933). *Biochem. J.* **27**, 1310.
—— Passmore and Peters (1932). *Proc. Roy. Soc. Lond.* B **111**, 291.
—— —— —— (1933). *Biochem. J.* **27**, 1872.
Passmore, Peters and Sinclair (1933). *Biochem. J.* **27**, 842.
Szent-Györgyi (1925). *Biochem. Z.* **157**, 50.
—— and Vargha (1932). *Z. physiol. Chem.* **210**, 228.

LXXXVII. THE TRANSMISSION OF VITAMIN A FROM PARENTS TO YOUNG IN MAMMALS.

III. EFFECT OF THE FAT CONTENT OF DIET DURING PREGNANCY ON THE TRANSMISSION OF VITAMIN A TO THE FOETAL RAT.

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In an earlier paper [Dann, 1932] a preliminary investigation of the factors controlling the transmission of vitamin A from mammalian mothers to their young was described, most of the work being done upon the rat. One of the conclusions reached, which is further examined in the present communication, was that "the amount of vitamin A in the liver of the foetal rat at birth is small and subject to little variation. It cannot be increased by giving large amounts of carotene to the mother before or during pregnancy." The provitamin A, carotene, was used in those experiments because at that time no rich concentrate of vitamin A was available in the quantities required. Since the publication of the earlier paper, however, concentrates of vitamin A have become available in large quantities, and it has been thought advisable to repeat the work upon the rat using vitamin A itself in the diet. The repetition (described below) led to a conclusion similar to that reached from the earlier work, namely that the amount of vitamin A in the liver of the foetal rat remains small even when the parent doe receives large amounts of vitamin A before or during gestation.

Having established this point, it remained to be seen whether other constituents of the diet exercised any marked effect on the amount of vitamin A transmitted to the foetus during gestation when there was a liberal amount of it in the doe's diet. A factor which might *a priori* be expected to exercise some effect is the fat content of the diet, and in the present paper an investigation of the effect of the fat content of the maternal diet on the transmission of vitamin A to the foetus is described.

Experimental.

The effect of a diet rich in vitamin A on its transmission to the foetus.

For this work piebald rats from the breeding colony of this laboratory were used. The sexes were segregated at weaning and all the animals kept on the stock diet for breeding animals, consisting of bread, milk, mixed corn, green stuff and meat. At 12 weeks old, the diet was changed to one made up to resemble the carotene-rich diet used in previous experiments [Dann, 1932], which consisted of maize meal 1060, whole milk powder 400, whole grain flour 200, yeast 200, wheat germ 60, calcium phosphate 60 and salt mixture 20; for each 90 g. of this basal

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diet 10 g. of red palm oil were allowed and mixed in. In place of the red palm oil for the present experiment was substituted the same weight of a 0·5 % solution of B.D.H. vitamin A concentrate in arachis oil. When the animals were 16 weeks old, bucks and does were placed together in a single large cage, and when the does were seen to be pregnant they were removed to separate cages but kept upon the same diet. At birth of the litters both parent and young were immediately killed and the liver of the doe was removed for estimation of its vitamin A content colorimetrically with antimony trichloride. The young were examined similarly in batches of four or five instead of singly, and the vitamin A content of the carcase after removal of the liver was also determined in every case to be negative. The results of the liver examinations are set out in Table I.

Table I. *Transmission of vitamin A to foetal rats, when the parent receives much vitamin A.*

Doe	1	2	3	4	5	6
Vitamin A content of doe's liver at parturition (in B.U.)	300,000	300,000	300,000	200,000	200,000	180,000
Vitamin A content of liver of foetus at birth (in B.U.)	25	33	30	20	35	20

The effect of the fat content of the diet.

For this work also our own stock breeding animals were used. At weaning the sexes were segregated and kept in large cages on the stock diet described above.

At the age of 16 weeks, when the experimental period began, the does were divided into two groups matched in weight, and each group was put into a single large cage, together with a suitable number of bucks of the same age. One group then received a diet poor in fat and the other group a diet rich in fat, each diet containing the same liberal amount of vitamin A. In order to ensure that these conditions were strictly observed, the diets were prepared as follows. A basal diet free from fat and vitamin A was composed of 600 g. maize meal, 200 g. skim milk powder, 200 g. dried yeast and 50 g. salt mixture; weekly batches of this diet were prepared. The two experimental diets were prepared fresh daily by the addition of the requisite quantities of arachis oil and vitamin A to the basal diet. A commercial concentrate of vitamin A (obtained from The British Drug Houses, Ltd.) was taken, and 1 g. was dissolved in 19 g. of arachis oil. Each day 1·5 g. of this were weighed out and mixed into 148·5 g. of the basal diet, giving an experimental diet containing 0·05 % of the vitamin A concentrate and 1 % of fat. A further 1·5 g. of the oil was weighed out and mixed with 28·5 g. of arachis oil; the 30 g. of dilute vitamin A solution were then mixed into 120 g. of the basal diet giving an experimental diet containing 0·05 % of the vitamin A concentrate and 20 % of fat. By adopting the procedure of daily preparation of diets in this way any change in the potency of successive batches of the vitamin A concentrate is prevented from contravening the conditions stipulated above, since the two diets on any one day will always contain the same percentage of vitamin A and one will always contain twenty times as much fat as the other.

When a doe was seen to be pregnant she was at once removed to a separate cage but kept on the same diet as she had previously received. At parturition the young were removed immediately and decapitated, and the carcases were digested in aqueous KOH, from which the vitamin A was removed by extraction with ether and estimated by the antimony trichloride reaction.

Two experiments performed in this way are reported here. In the first, twelve piebald does were used; in the second, eight albino does. The results are collected in Tables II and III.

Table II. *Transmission of vitamin A to foetal piebald rats. The diet of each doe during gestation contained 0·05 % of vitamin A concentrate, but some received 1 % and others 20 % of fat.*

Doe	1	2	3	4	5	6	7	8	9	10	11	12
Fat content of doe's diet during gestation	20 %	20 %	20 %	20 %	20 %	20 %	1 %	1 %	1 %	1 %	1 %	1 %
Number of young in litter	9	8	8	10	6	9	12	8	8	10	8	8
Vitamin A store of each foetus (in B.U.)	31	25	33	35	37·5	50	25	33	25	19	25	25
Total amount of vitamin A transmitted to the whole litter (in B.U.)	279	200	264	350	225	450	300	264	200	190	200	200

Table III. *Transmission of vitamin A to foetal albino rats. The diet of each doe during gestation contained 0·05 % of vitamin A concentrate, but some received 1 % and others 20 % of fat.*

Doe	1	2	3	4	5	6	7	8
Fat content of doe's diet during gestation	20 %	20 %	20 %	20 %	1 %	1 %	1 %	1 %
Number of young in litter	9	9	12	7	6	10	10	9
Vitamin A store of each foetus (in B.U.)	11·5	37	46	16	21	30	16	9
Total amount of vitamin A transmitted to the whole litter (in B.U.)	104	243	552	112	186	300	160	81

Discussion.

The first part of the experimental work described shows that the amount of vitamin A transmitted to the foetal rat during gestation is still strictly limited when the parent animal has an enormous reserve of vitamin A in her own liver and receives a diet rich in vitamin A during gestation. The amount of vitamin A found in the foetal rats at birth was however greater in these experiments than in the author's previous experiments where the provitamin carotene was included in the diet of the parent, but it must be borne in mind that in the present experiments the does received at a moderate estimate five thousand times the necessary minimum of vitamin A in their diet.

Turning to the effect of fat in the maternal diet, it can be seen from the figures collected in Table II that the average amount of vitamin A found in the foetal piebald rats at birth was 35 B.U. when the doe received during gestation a diet containing 20 % of fat and 0·05 % of vitamin A concentrate, and 25 B.U. when the doe received a diet containing 1 % of fat and 0·05 % of vitamin A concentrate. The average figures for the total amounts of vitamin A transmitted to the whole litter during gestation were 294 B.U. and 226 B.U. for does receiving high fat and low fat diets respectively. From Table III the average figures for the albino rats are 27 B.U. and 19 B.U. transmitted to each foetus, and 253 B.U. and 182 B.U. transmitted to the whole litters, on high fat and low fat diets respectively.

From these figures it appears that an increase in the proportion of fat in the diet of a pregnant doe will in general cause an increased transmission of vitamin A to the foetus. It is plain however that with the wide differences in fat content now tested, the variation in the amount transmitted is so small (though definite) that the effect of ordinary variations in the fat content can be neglected when the diet is rich in the vitamin.

SUMMARY.

1. When the female rat receives large amounts of vitamin A (of the order of 5000 times the necessary minimum) in her diet before and during gestation, her young have only a small store of vitamin A in their livers at birth. The amount is generally between 20 and 35 B.U. of the vitamin. In the body apart from the liver no vitamin A can be detected.

2. The amount of vitamin A transmitted to the foetal rat during gestation when the parent doe receives large amounts in her diet is affected by the fat content of the diet and is increased as the fat content increases. This increase in transmission is small and for ordinary ranges of variation of fat content is probably negligible.

I wish to express my thanks to Dr T. Moore for his interest in this work and for many suggestions towards its furtherance.

REFERENCE.

Dann (1932). *Biochem. J.* **26**, 1072.

LXXXVIII. ASCORBIC ACID IN THE EYE-LENS AND AQUEOUS HUMOUR OF THE OX.

BY THOMAS WILLIAM BIRCH AND WILLIAM JOHN DANN[1].

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(*Received March 2nd, 1934.*)

IN an earlier communication [Birch and Dann, 1933] on the estimation and distribution of ascorbic acid and glutathione in animal tissues we drew attention to the distribution of ascorbic acid in organs other than the suprarenal cortex, as detected by the indophenol reagent. The presence in a number of tissues of reducing substances other than glutathione had become evident from the work of Mason [1930] and of Bierich and Rosenbohm [1933], but no evidence of the identity of such substances had been obtained. By applying the indophenol titration for ascorbic acid to tissue extracts we were able to demonstrate the presence of the acid in a number of organs and to give figures for the amounts present. At the same time it was pointed out that the indophenol titration may not be specific for ascorbic acid in tissue extracts, so that other tests are necessary to confirm the results obtained by the titration method.

The silver nitrate staining test was applied to a number of the tissues which had been found by means of the indophenol titration to contain ascorbic acid, with results which were not always in harmony with those obtained by the latter method. Some tissues found by the titration method to contain ascorbic acid gave little or no staining with silver nitrate, and led us to the conclusion already formulated by Harris and Ray [1933, 1] that a positive result with this test indicates the presence of ascorbic acid in a tissue but a negative result does not necessarily mean that ascorbic acid is absent.

In order to examine the reliability of results obtained by the titration method it was decided to carry out biological assays of the ascorbic acid found by the titration method to occur in unexpected sites. The tissues chosen for examination were the eye-lens and aqueous humour of the ox.

EXPERIMENTAL.

For the first attempt to assay biologically the ascorbic acid in the lens and the aqueous humour of the ox's eye the recovery test of Harris *et al.* [1932] was employed. Young guinea-pigs fed on a full diet of natural foodstuffs on attaining the weight of 300 g. were placed on a vitamin C-free diet consisting of bran 80 parts, ground oats 720, egg-yolk 40, salts 84, cod-liver oil 1 %, and were weighed daily thereafter. As soon as any animal showed a loss in weight on two successive days, it was given its first dose of the material under test and then dosed daily. The weight curves of the animals are shown in Fig. 1.

Since no simple method of extracting the ascorbic acid from the lens could be devised, the method of preparing the lens for dosing was to remove the lenses

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from a number of ox eyes which had been kept in cold storage after the slaughtering and to estimate by the indophenol micro-titration the amount of ascorbic acid in a sample of the lenses. Weighed amounts of the lenses calculated to contain (from the titration results) either 1 mg. or 3 mg. of ascorbic acid were then mashed up in a little water until the suspension could be taken up in a pipette and given to the guinea-pig. For doses of the aqueous humour, the humour was extracted from a number of eyes and pooled; an ascorbic acid

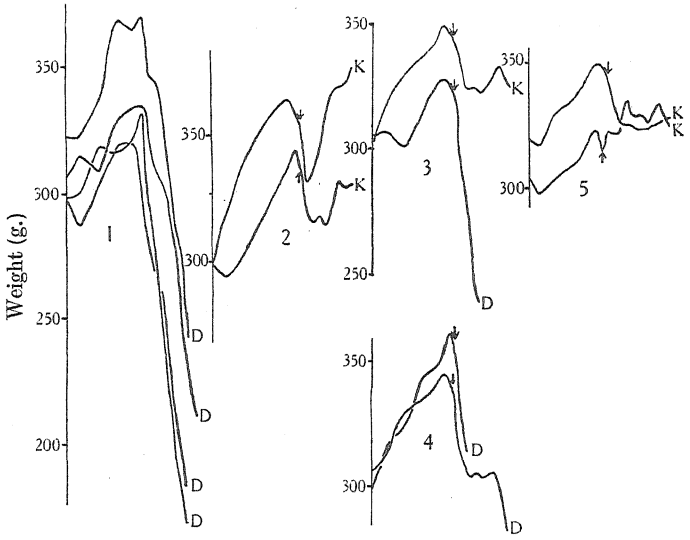

Fig. 1. Weight curves of guinea-pigs used in recovery test. The daily doses of the different groups were as follows: (1) No addition to basal diet; (2) 3·0 ml. of orange juice; (3) Eye-lens calculated from indophenol titration to contain 1 mg. ascorbic acid; (4) Eye-lens calculated to contain 3 mg. ascorbic acid; (5) Aqueous humour calculated to contain 2 mg. of ascorbic acid. Dosing commenced as indicated by the arrows. D signifies died; K signifies killed.

determination was then made on a portion of the mixed humour. The amount of humour calculated to contain 2 mg. ascorbic acid was then given to the guinea-pig from a pipette. The ascorbic acid estimation and preparation of doses were carried out each day, and a fresh supply of eyes was obtained as often as possible (generally every day).

From the weight curves in Fig. 1 it can be seen that the test was not successful. Three of the four animals receiving a supplement of eye-lens died before the first of the negative control animals, the average time between commencement of the experimental period and death being $4\frac{1}{4}$ days for the animals receiving eye-lens and $6\frac{1}{2}$ days for the negative controls. Thus no evidence of the presence of ascorbic acid in the lens is afforded by this test, which however suggests not so much the absence of ascorbic acid from the lens as the presence of some other substance exerting a positive toxic effect. The animals receiving aqueous humour lived through the experimental period of 9 days but did not show an increase of weight, as did the positive controls receiving 3 ml. of orange juice daily. The curves indicate that the dose of aqueous humour contained about 1·2 mg. ascorbic acid, or about two-thirds of the amount found by the titration. If the aqueous humour exerts in slighter degree a similar toxic action to the lens, then

the amount of ascorbic acid present would be greater than is indicated by the curves.

On account of the failure of this test with the lens a second test was arranged with alterations considered likely to diminish the effect of the toxic action of the lens. Instead of the curative test, the tooth structure method of Höjer as modified by Key and Elphick [1931] was used, as it has the advantage that at the beginning of the dosing period the guinea-pigs have not been entirely depleted of their vitamin C and therefore are likely to be more resistant to the toxic action of the dosing material. For convenience of dosing a large batch of ox eyes was obtained, and the lenses were removed, dried in a vacuum-desiccator and powdered. The dried powdered lenses were well ground and mixed to give a uniform material, which by titration against the indophenol was found to contain 0·9 mg. ascorbic acid per g. The indophenol reagent was standardised against pure ascorbic acid. The dose given to each animal daily was 3 g. of the powdered eye-lens, containing according to the titration 2·7 mg. of ascorbic acid. Four animals were used, together with three negative controls, four animals receiving 3·5 ml. orange juice daily and three animals receiving 2 ml. orange juice daily. After 14 days all the animals were killed and their lower jaws dissected out for examination of the teeth. During the dosing period one animal receiving the eye-lens had gained a little in weight, one had remained steady and the other two had fallen considerably. The results of the histological examination of the teeth are set out in Table I.

Table I.

Guinea-pig no.	Daily dose	Degree of protection from scurvy*	
1	3 g. dried eye-lens	2·6	
2	,,	2·2	Mean 2·5
3	,,	2	
4	,,	3·3	
5	3·5 ml. orange juice	2·3	
6	,,	3·5	Mean 3·2
7	,,	3	
8	,,	3·8	
9	2 ml. orange juice	1·5	
10	,,	1	Mean 1·4
11	,,	1·8	
12	None	0	
13	,,	0·3	Mean 0·5
14	,,	1·2	

* Each figure in this column is the mean of three, allotted by three independent observers.

From the figures given it appears that ascorbic acid is undoubtedly present in the lens of the ox eye. The doses of orange juice given to the positive control animals were chosen to contain 2·1 mg. and 1·2 mg. of ascorbic acid in 3·5 ml. and 2·0 ml. respectively. Since this work was undertaken, however, it has been demonstrated by Harris and Ray [1933, 2] that the ascorbic acid content of the juice of different oranges varies a good deal more than was supposed. Hence it is difficult to give a quantitative figure for ascorbic acid content of a test dose used in a biological test unless the positive controls have been dosed with pure ascorbic acid. On the assumption that the orange juice used in our experiments was near to the average in ascorbic acid content (*i.e.* 0·6 mg. per ml. of juice) then the results set out in Table I indicate that the eye-lens contains ascorbic acid to the extent of about two-thirds of the amount suggested by indophenol titration.

SUMMARY.

The eye-lens and aqueous humour of the ox have been tested biologically by the tooth protection method and the recovery method respectively, and the presence of ascorbic acid found by the indophenol titration method has been confirmed.

REFERENCES.

Bierich and Rosenbohm (1933). *Z. physiol. Chem.* **215**, 151.
Birch and Dann (1933). *Nature*, **131**, 469.
Harris, Mills and Innes (1932). *Lancet*, **223**, 235.
—— and Ray (1933, 1). *Biochem. J.* **27**, 2006.
—— —— (1933, 2). *Biochem. J.* **27**, 2016.
Key and Elphick (1931). *Biochem. J.* **25**, 888.
Mason (1930). *J. Biol. Chem.* **86**, 623.

LXXXIX. FURTHER STUDIES ON THE LACTIC ACID, SUGAR AND INORGANIC PHOSPHORUS OF THE BLOOD OF RUMINANTS, (*a*) FOLLOWING ADRENALECTOMY AND (*b*) AFTER INTRAVENOUS INJECTION OF INSULIN.

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(*Received March 3rd, 1934.*)

In a previous paper [Allcroft and Strand, 1933] we have shown that a marked increase occurs in the percentages of lactic acid and sugar in the blood of sheep after muscular exercise and/or excitement, and it seemed important to determine whether these changes would also occur after adrenalectomy. While there are numerous references to the effect of adrenalectomy in dogs and small laboratory animals, we can find only one reference to similar work in the ruminant. Moore and Purinton [1901], working on two goats and two kids, found that the operation was fatal in three out of the four cases.

Briggs *et al.* [1923–24] state that after insulin injections in normal animals, there is a decrease in glucose, phosphorus and potassium in the blood with an increase in blood-lactic acid. Best and Ridout [1925] state that the lactic acid content of the blood of dogs does not significantly increase during insulin hypoglycaemia. Cori [1925] reports that insulin hypoglycaemia was attended by no definite changes in the lactic acid of either fasting rabbits or cats: later, however [1931; 1932], this worker reports an increase in lactic acid and a decrease in phosphorus after injection of insulin in normal rabbits, but after adrenalectomy these changes were not observed. Katayama and Killian [1926–27] also conclude that insulin produces an increase in the lactic acid of the blood of rabbits and human beings and note that in the rabbit the changes in concentration of lactic acid are very slight in comparison with the drop in blood-sugar. These rather contradictory reports led us to attempt to study the changes in lactic acid, sugar and inorganic phosphorus of the blood of sheep after injection of insulin. Though it was not possible to complete the study it is thought advisable to report the observations which were made.

Technique.

The sheep were brought in from grazing and put in metabolic cages for 18–24 hours before operation. Food was withheld during this period but water was available. The animals were anaesthetised by ether and the wool was cut short, but not shaved, over the skin of the abdomen between the costal margin and the umbilicus. An oblique incision (about 8 in. long) was then made in the loin stretching from the lower border of the 12th rib on the right side

towards the umbilicus. By retraction of the viscera, the adrenal gland was readily exposed and the small vein crossing in front of it from the parietes to the inferior vena cava was doubly ligated. Narrow tapes were then placed with care under the vena cava above and below the site of the gland. By gently pulling on these it was possible to control the bleeding during the removal of the gland, which was the next step. After removal, any obvious small veins or arteries were tied and the longitudinal slit which is unavoidably made in the vena cava, as a result of the excision of the gland (on account of the short length of the adrenal vein) stitched by fine linen thread on fine needles, and a small piece of wet cotton wool placed over the suture line to prevent leakage. The abdominal wound was then closed and the animal allowed to recover from this first operation. The removal of the left adrenal gland followed in 8–20 days. In this case the length of the adrenal vein was sufficient to permit of the removal of the gland without damaging the vena cava. The animals recovered well from both operations, without any suppuration of the wounds.

In a few cases cortico-adrenal extract was injected after bilateral adrenalectomy to maintain the animal in good condition. The extract was made by Grollman and Firor's method [1933] and assayed by intraperitoneal injection into month-old adrenalectomised rats. If the extract were potent the rats gained 1–4 g. in weight daily and appeared to be in a normal condition. Occasional batches of extract were not potent, and after injection of these the rats lost weight daily showing typical adrenal insufficiency symptoms such as loss of appetite, muscular weakness, general apathy and often convulsions just before death, which resulted in 2–4 days after the operation.

Although the extracts were as a rule potent as judged by their effect on growing rats, it is not certain, owing to the limited number of experiments, whether their injection into adrenalectomised sheep really prolonged life (see Table I). We gained the impression however that the injected animals were in better condition than the controls.

Owing to the short survival period of bilaterally adrenalectomised sheep it was found necessary to carry out the excitement experiments as soon as possible and the second day after the final operation was chosen since by that time the blood-sugar had assumed a low level. Blood samples were taken from most of the animals before unilateral adrenalectomy, in the period between unilateral and complete adrenalectomy, and after the final operation. The technique and times of sampling were essentially the same as in the earlier work except that sampling was continued for only 2 hours following the excitement, since this period covered the major changes which we had noted.

In the insulin injection experiments, insulin was injected into the left jugular vein and samples of blood were taken from the right jugular vein, before injection and at intervals of 15, 30, 45, 60, 90, 120, 180, 240 and 300 minutes afterwards. The amount of insulin injected per kg. body weight is given in each graph. In the case of the one adrenalectomised sheep to which insulin was administered it was not possible to obtain the full series of samples owing to convulsions, which occurred 50 minutes after the insulin injection.

Lactic acid was estimated by the method of Friedemann *et al.* [1927], blood-sugar by the Hagedorn and Jensen [1920] method and inorganic phosphorus by Briggs method [1922]. For all estimations the blood-proteins were precipitated within 1–2 minutes after the blood sample was drawn.

The results of parts (*a*) and (*b*) are given and discussed separately.

(*a*) The following table (Table I) gives the results of adrenalectomy on the blood constituents.

Table I.

Results expressed in mg./100 ml. whole blood.

Sheep No.	Before unilateral adrenalectomy			After unilateral adrenalectomy			After complete adrenalectomy			Survival period in (hours)	Remarks
	Lactic acid	Blood-sugar	Inorg. P	Lactic acid	Blood-sugar	Inorg. P	Lactic acid	Blood-sugar	Inorg. P		
167	15·23	61·80	—	10·40	57·40	—	5·80	45·96	8·44	52	Left gland removed 8 days after right. In good condition first day after complete adrenalectomy. Insufficiency symptoms set in about 9 a.m. second day. No C-A extract injected
	15·57	60·04	—	10·12	61·80	7·76	7·00	40·68	8·64		
	—	—	—	7·72	61·80	7·76	6·05	35·40	9·64		
	—	—	—	—	—	—	8·95	40·68	9·64		
125	—	—	—	22·05	79·40	—	8·45	35·40	—	58	Left gland removed 10 days after first operation. In good condition first day after complete adrenalectomy. On second day insufficiency symptoms were marked. No C-A extract injected
	—	—	—	19·50	81·16	—	6·26	35·40	—		
	—	—	—	20·20	—	—	8·45	37·16	10·72		
	—	—	—	—	—	—	8·45	34·52	11·64		
1	17·45	55·64	3·94	—	—	—	—	—	—	—	Left gland removed 7 days after first operation. Died a few hours after second operation due to haemorrhage from inferior vena cava
	15·50	52·12	4·21	—	—	—	—	—	—		
	13·30	52·00	4·47	—	—	—	—	—	—		
2	47·70	68·84	6·08	—	—	—	(1) 11·10	41·56	10·64	34	Left gland removed 8 days after right. In good condition day after complete adrenalectomy. Two blood samples taken 9.30 and 10 a.m. (Nos. 1 and 2). Then 10 ml. of C-A extract injected intraperitoneally. Two more samples (3 and 4) taken 15 and 30 minutes after injection. No insufficiency symptoms apparent but sheep died a few hours after injection of toxic extract No. 5*
	17·70	73·24	5·42	—	—	—	(2) 13·75	44·20	9·86		
	27·90	74·12	5·42	—	—	—	(3) 13·75	65·32	10·80		
	—	—	—	—	—	—	(4) 14·50	66·20	11·16		
3	15·00	71·48	6·03	12·30	64·44	4·42	7·60	47·72	7·30	60	Left gland removed 18 days after first operation. In good condition next day and following day during which it received 30 ml. extract in 5 ml. injections. Was in good condition 6.30 p.m. on second day but collapsed suddenly and died 11 p.m. same night
	13·05	74·12	5·32	19·00	60·04	5·09	6·40	38·92	8·14		
	14·25	73·24	5·44	20·50	66·20	5·17	7·25	43·32	7·89		
4	18·30	61·80	5·53	11·80	62·68	2·79	—	—	—	—	Left gland removed 16 days after first operation. Died the morning following the second operation due to tying of the vena cava
	11·10	62·68	4·98	9·70	65·32	2·79	—	—	—		
	12·56	62·68	4·66	10·40	68·84	2·56	—	—	—		
5	24·00	70·60	5·65	—	—	—	(1) 11·40	54·76	7·94	52	Left gland removed 6 days after right. 5 ml. C-A extract (No. 11) injected at hourly intervals during day following complete adrenalectomy. Animal in very good condition. Samples 1 and 2 taken day after second operation and samples 3 and 4 the next morning. After No. 4 sample was taken insulin (0·75 units/kg. body weight) was injected. 50 minutes afterwards animal had convulsions. C-A extract and large quantities of glucose solution were injected but sheep did not recover and died 7 hours after insulin injection
	26·70	69·72	5·60	—	—	—	(2) 11·60	54·76	7·68		
	26·00	70·60	5·60	—	—	—	(3) 12·15	62·68	12·24		
	—	—	—	—	—	—	(4) 10·40	55·64	13·00		

* This extract was found to be highly toxic by injecting 0·5 ml. of it intraperitoneally into mice.

The effects of excitement are shown in the curves of Figs. 1–4.

Sheep Nos. 167 and 125 (Figs. 1 and 2) were subjected to a barking dog but showed singular lack of interest in the proceedings. After the 120 minute sample had been taken one of the sheep (No. 125) was exercised for 5 minutes and then samples taken immediately and 15 minutes after. The dog barking caused no significant alteration in the level of any of the three constituents studied in

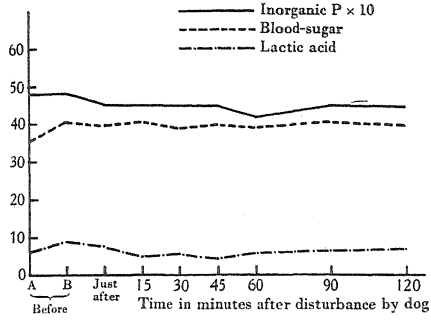

Fig. 1.

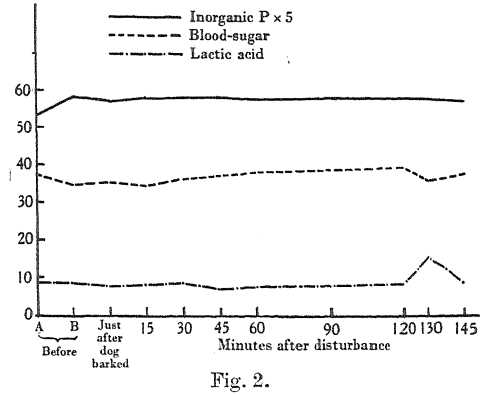

Fig. 2.

Fig. 1. Effect of excitement (dog barking) on sheep No. 167 bilaterally adrenalectomised. Ordinate: mg. per 100 ml. whole blood.

Fig. 2. Effect of excitement (dog barking) on sheep No. 125 bilaterally adrenalectomised. 10 minutes' exercise between 120 and 130 minutes. Ordinate: mg. per 100 ml. whole blood.

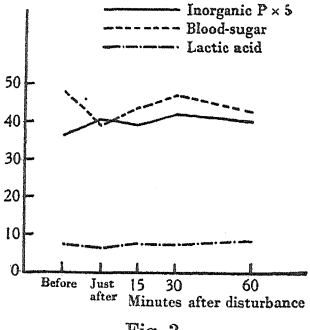

Fig. 3.

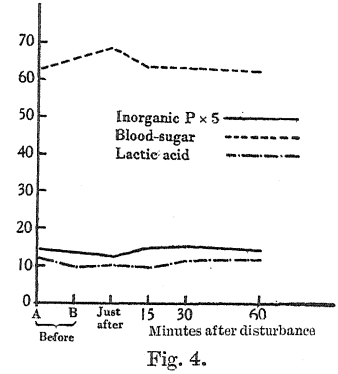

Fig. 4.

Fig. 3. Effect of excitement (by explosion of cracker) on sheep No. 3 bilaterally adrenalectomised. Ordinate: mg. per 100 ml. whole blood.

Fig. 4. Effect of excitement (by explosion of cracker) on sheep No. 4 unilaterally adrenalectomised. Ordinate: mg. per 100 ml. whole blood.

either animal, and the changes in lactic acid and sugar which occurred in sheep No. 125 after exercise are so small as to be of doubtful significance (Fig. 2).

Sheep Nos. 3 and 4, the former bilaterally, and the latter unilaterally adrenalectomised, were subjected to the explosion of a medium-sized firework placed about 4 ft. from them. Sheep No. 3 (Fig. 3) showed no alteration in lactic acid or inorganic P but the curve for sugar showed a lower level after the explosion, returning to the pre-experimental level within 30 minutes. In sheep

No. 4 the only change was a slight elevation of the blood-sugar immediately after the explosion (Fig. 4). This increase in blood-sugar and the normal low level of the inorganic P is, in our opinion, due to the fact that the animal is not completely adrenalectomised.

Discussion.

(*a*) It can be seen from the above results that the blood-lactic acid was lower after complete adrenalectomy than before or after unilateral adrenalectomy. This is contrary to the conclusions of Britton and Silvette [1932] with cats, but in agreement with those of Hastings and Compere [1930–31] with dogs. The blood-sugar level was also considerably lowered after complete adrenalectomy. Injection of C-A extract definitely raised the blood-sugar in one sheep, No. 2, and possibly diminished the fall in another, No. 5 [*cf.* Britton and Silvette, 1932; Wyman and Walker, 1929].

The inorganic P was considerably raised after complete adrenalectomy. It was observed too in every case after the second gland was removed that there was a marked increase in the blood concentration so that it was often difficult to get a series of blood samples. Just before death the usual symptoms of adrenal insufficiency were present, *viz.* refusal of food, muscular weakness, prostration, decrease in body temperature and finally convulsions. These observations, increase in inorganic P, anhydraemia and insufficiency symptoms, agree with those made by Britton and Silvette [1932], Britton [1932] and Lucas [1926].

Banting and Gairns [1926] state that the lowering of the blood-sugar does not depend on the absence of the adrenals but is the result of the shock of the operation. If this were so one would expect a gradual rise in the blood-sugar as the animal recovered from the operation, but in no instance was this observed. None of the animals survived for a longer period than 60 hours after complete adrenalectomy. In the cases where cortico-adrenal extract was injected (except in sheep No. 5) the short survival period was probably due to the fact that the extract was not sufficiently potent to prolong the life of the animal. On the same extract however we were able to keep completely adrenalectomised month-old and adult rats indefinitely. It is extremely probable that sheep No. 5 died of insulin shock since it has been shown that adrenalectomy renders other animals hypersensitive to insulin [Ellsworth and Weinstein, 1933; Tada, 1931; Lewis and Magenta, 1925; Houssay and Lewis, 1923]. Moore and Purinton [1901] record survival periods for two bilaterally adrenalectomised kids of 49 and 60 hours, while one goat survived 8 days and in another the results were not fatal.

Effect of excitement. It is evident that in completely adrenalectomised sheep no significant alteration occurs in the levels of lactic acid, sugar or inorganic P of the blood after the same type of emotional stimuli which were found to cause marked rises in normal sheep [Allcroft and Strand, 1933]. This confirms the views of Lumley and Nice [1930], who showed that after excitation induced by shocks sufficient to cause struggling in adrenalectomised rats, no increase in the blood-sugar occurred, while in normal rats under similar conditions increases up to 86 % were observed. Britton [1933] also reports that none of the measures which normally produce hyperglycaemia in cats, *e.g.* adrenaline, anaesthesia or excitement, has any significant influence on the blood-sugar level of adrenalectomised animals.

(*b*) Figs. 5–8 show graphically the changes which occurred after insulin injection in the four unoperated sheep. Figs. 5 and 7 show results obtained on the same sheep with an interval of 16 days between the experiments.

In Table II are given the results of the few blood samples which were secured from the completely adrenalectomised sheep, and also in chronological sequence the therapeutic treatment administered during the convulsive stages. Though

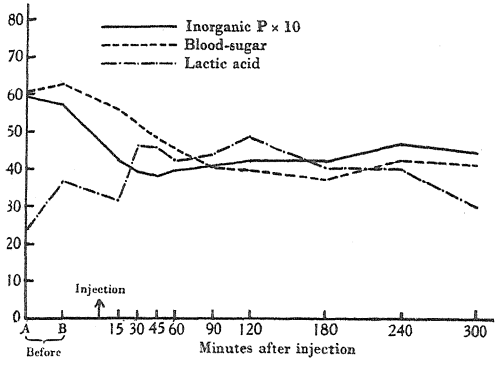

Fig. 5.

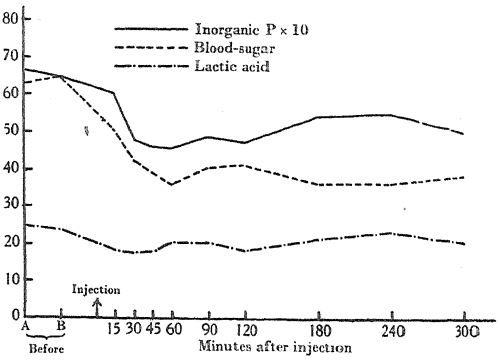

Fig. 6.

Fig. 5. Effect of intravenous injection of insulin (2 units per kg.) on normal sheep No. 9. 25. ix. 33. Ordinate: mg. per 100 ml. whole blood.

Fig. 6. Effect of intravenous injection of insulin (2 units per kg.) on normal sheep No. 13. 25. ix. 33. Ordinate: mg. per 100 ml. whole blood.

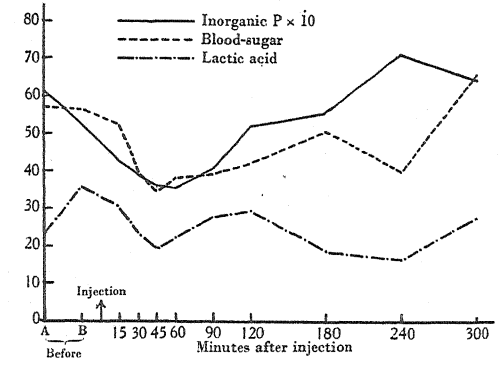

Fig. 7.

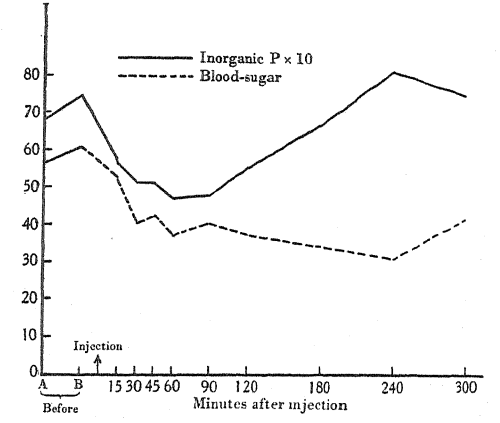

Fig. 8.

Fig. 7. Effect of intravenous injection of insulin (2 units per kg.) on normal sheep No. 9. 11. x. 33. Ordinate: mg. per 100 ml. whole blood.

Fig. 8. Effect of intravenous injection of insulin (3 units per kg.) on normal sheep No. 10. 11. x. 33. Ordinate: mg. per 100 ml. whole blood.

the animal had been maintained in good condition with cortico-adrenal extract up to the commencement of the experiment the subsequent injections of glucose and cortico-adrenal extract were unable to prevent death 7 hours after injection of insulin.

It will be seen that in only one case (Fig. 5, sheep No. 9) did the blood-lactic acid show any definite increase after intravenous injection of insulin. The samples obtained at 45 and 300 minutes after injection of insulin into the adrenalectomised sheep (see Table II) gave high values for lactic acid associated

Table II. *Effect of insulin injection on a completely adrenalectomised sheep.*

27·2 kg. weight (0·75 unit insulin per kg.) 20·4 units injected.
Results expressed in mg./100 ml. whole blood.

	Lactic acid	Blood-sugar	Inorganic P	Remarks
Before injection	12·15	62·68	12·24	—
	10·40	55·64	13·00	Injection of 0·75 unit insulin per kg. body weight
15 mins. after	11·60	45·96	12·16	—
30 „	10·05	40·68	11·54	—
45 „	23·75	36·28	12·50	—
50 „	—	—	—	Convulsions began. 11 ml. glucose intravenously
70 „	—	—	—	11 ml. glucose intravenously
83 „	—	—	—	11 ml. glucose intravenously
95 „	—	—	—	11 ml. glucose intravenously + 0·5 ml. adrenaline subcutaneously
210 „	—	—	—	60 ml. glucose subcutaneously
300 „	20·10	57·40	10·98	—

with the hypoglycaemic convulsions. The changes in the blood-sugar correspond to those reported in the literature, although the degree of hypoglycaemia produced was not the same in all cases, and the low level seemed more prolonged in the ruminant than in simple-stomached animals. The fall in inorganic P agrees with the work of Cori and Cori [1932] on rabbits and of Eadie *et al.* [1925] on dogs and rabbits. The former observed that the level was lowest 45 minutes after injection and gradually returned to the original level. Eadie *et al.* [1925] state that the blood-phosphate fall is simultaneous with that of blood-sugar, but the rise to normal occurs before that of sugar and in some cases may continue above the normal level. In our own work the lowest levels lie between 45 and 60 minutes after injection and in two cases (Figs. 7 and 8) there was a rise to above the normal level. It is possible that this return to a higher level was delayed in Figs. 5 and 6 and might have occurred after the last samples were taken. Ellsworth and Weinstein [1933] observed similar phenomena in dogs whether normal, or completely adrenalectomised and maintained on cortical extract: one adrenalectomised sheep however showed only a very slight fall in inorganic P.

It is obvious that adrenalectomy increases the sensitivity of the sheep to insulin just as it does that of other animals. At the same time it is evident that the normal ruminant can tolerate much larger doses of insulin without occurrence of convulsions than can simple-stomached animals. Peterson *et al.* [1931] report that they lowered the blood-sugar in cows to values as low as 17 mg. per 100 ml. by insulin injections and yet failed to induce convulsions.

Summary.

1. A description is given of the surgical technique used for adrenalectomy operations on sheep.

2. After bilateral adrenalectomy in sheep the blood-sugar and lactic acid levels were definitely lowered while that of inorganic P was raised.

3. No change occurred in blood-sugar and lactic acid of adrenalectomised sheep when they were subjected to such types of excitement and muscular exercise as were known to raise them in normal sheep.

4. There was no definite change in the blood-lactic acid of normal sheep after insulin injections.

5. The hypoglycaemia produced by insulin injection in normal ruminants was similar to that in other animals except that no sign of convulsions was observed even after large doses of insulin.

6. The changes in blood-inorganic P after insulin corresponded to those found in other animals.

7. Bilateral adrenalectomy increases the sensitivity of the ruminant to insulin.

We wish to express our thanks to Prof. J. J. R. Macleod for his advice and interest in this work, to Dr R. A. Cleghorn and Dr H. E. Magee for assistance in part of it.

REFERENCES.

Allcroft and Strand (1933). *Biochem. J.* **27**, 512.
Banting and Gairns (1926). *Amer. J. Physiol.* **77**, 100.
Best and Ridout (1925). *J. Biol. Chem.* **63**, 197.
Briggs (1922). *J. Biol. Chem.* **53**, 13.
—— Koechig, Doisy and Weber (1923–24). *J. Biol. Chem.* **58**, 721.
Britton (1932). *Amer. J. Physiol.* **99**, 9.
—— (1933). *Amer. J. Physiol.* **105**, *Proc.* 11.
—— and Silvette (1932). *Amer. J. Physiol.* **100**, 701.
Cori (1925). *J. Biol. Chem.* **63**, 253.
—— (1931). *Physiol. Rev.* **11**, 143.
—— and Cori (1932). *J. Biol. Chem.* **97**, *Proc.* lxxxv.
Eadie, Macleod and Noble (1925). *Amer. J. Physiol.* **72**, 164.
Ellsworth and Weinstein (1933). *Bull. Johns Hopkins Hosp.* **53**, 21.
Friedemann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 335.
Grollman and Firor (1933). *J. Biol. Chem.* **100**, 429.
Hagedorn and Jensen (translation by Host and Hatlehol) (1920). *J. Biol. Chem.* **42**, 347.
Hastings and Compere (1930–31). *Proc. Soc. Exp. Biol. Med.* **28**, 376.
Houssay and Lewis (1923). *Compt. Rend. Soc. Biol.* **89**, 1120.
Katayama and Killian (1926–27). *J. Biol. Chem.* **71**, 707.
Lewis and Magenta (1925). *Compt. Rend. Soc. Biol.* **92**, 821.
Lucas (1926). *Amer. J. Physiol.* **77**, 114.
Lumley and Nice (1930). *Amer. J. Physiol.* **93**, 152.
Moore and Purinton (1901). *Amer. J. Physiol.* **5**, 182.
Peterson, Hewitt, Boyd and Brown (1931). *J. Amer. Vet. Med. Assoc.* **79**, 217.
Tada (1931). *Tôhoku J. Exp. Med.* **17**, 588.
Wyman and Walker (1929). *Amer. J. Physiol.* **89**, 215.

XC. THE ULTRAFILTRATION OF PROTEINS THROUGH GRADED COLLODION MEMBRANES.

I. THE SERUM-PROTEINS.

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Many qualitative investigations showing the influence of various physical factors upon the process of ultrafiltration are contained in the literature. Elford [1933] has discussed these effects in a more quantitative manner, based on experimental results with graded collodion membranes [Elford, 1931]. The latter afford a unique advantage in that they provide a wide selection of grades and can be accurately calibrated in terms of average pore diameter; and groups of membranes known to be of the same grade may be used in comparative filtrations with different systems. The present study considers the influence upon the ultrafiltration of proteins of the nature of the solvent medium, its hydrogen ion concentration, the concentration of protein and filtration pressure. It establishes a firmer basis for the standard conditions which have been adopted as being most favourable for ultrafiltration studies on viruses and bacteriophages in this Laboratory (for a partial bibliography see Burnet and Andrewes [1933]). It confirms the values previously presented for the empirical correction factor used in estimating the particle size of a virus or phage from the pore diameter of the limiting membrane which, under those optimum conditions, just completely retains it.

Materials.

The proteins used were highly purified samples of horse serum-albumin and pseudoglobulin, kindly presented to us by Dr H. W. Dudley, to whom we acknowledge our indebtedness. The pseudoglobulin was prepared by precipitation from normal horse serum with half-saturated ammonium sulphate, and subsequent dissolution in water and precipitation with ammonium sulphate three times. The final precipitate was dissolved in water and dialysed under slight pressure. This served to precipitate any remaining euglobulin, which was sedimented by centrifuging. The clear centrifugate, containing the pseudoglobulin, was concentrated *in vacuo* over sulphuric acid to obtain the solid protein. The albumin, contained in the first filtrate from the globulin precipitation, was precipitated three times by the method of Hopkins and Pinkus [1898], the requisite amount of acetic acid for producing a crystalline precipitate being determined in each case by trial. The final precipitate was redissolved in water and dialysed and concentrated *in vacuo* as above.

When the albumin was dissolved in water, or the pseudoglobulin in 0·05 % sodium chloride solution, practically no insoluble residue remained. Protein-free ultrafiltrates of solutions of both proteins in distilled water contained no chloride

detectable by the silver nitrate test and only a trace of sulphate as detected by barium chloride. The ash contents of the solid samples were: albumin, 0·8 %; pseudoglobulin, 0·4 %.

Procedure.

Exclusive use was made of the graded collodion ("gradocol") membranes, prepared and calibrated by the methods of Elford [1931] and Elford and Ferry [1934]. Nickel-plated ultrafilters of the type described by Barnard and Elford [1931] were slightly attacked by the more acidic protein solutions, and accordingly silver-plated models were employed. The components of the apparatus were sterilised separately and assembled under sterile conditions. Filtration was carried out under positive pressure of from 1 to 3 atmospheres of nitrogen. In comparative experiments, six filtrations could be run simultaneously from a pressure distributor attached to one nitrogen cylinder.

In a typical experiment, a solution of protein was made up to the desired concentration in a given solvent medium, and the p_H was adjusted, if necessary, by addition of small quantities of 0·1 *N* or *N* hydrochloric acid or sodium hydroxide. The solution was then filtered through a membrane of grade between 0·5 μ and 1·0 μ to sterilise it. Filtration through such a membrane affords a very convenient method for removing bacteria and gross aggregates with minimum loss of protein by adsorption. This sterile stock filtrate could be kept for months at 4° without any appreciable change in appearance, p_H (unless originally alkaline, when absorption of CO_2 from the atmosphere soon shifted the reaction), or filtrability. In no case was an antiseptic preservative necessary. When a comparison of different media was required, the stock was made up to twice the final concentration desired, and aliquot parts were diluted with the different media.

Different samples, usually 10 ml. of stock solution, were filtered through various membranes, all of comparable thicknesses and of grades ranging from that which let the protein through in nearly undiminished concentration to that which yielded filtrates giving a negative test with sulphosalicylic acid. For each individual filtration, the filtrate was collected in successive ml. samples and analysed with a Zeiss dipping refractometer. The concentrated residue remaining over the membrane was also analysed. The refractometric analyses were roughly checked by precipitation with sulphosalicylic acid, which was sensitive to a concentration of about 0·005 % protein. The relative concentration, *u*, of the

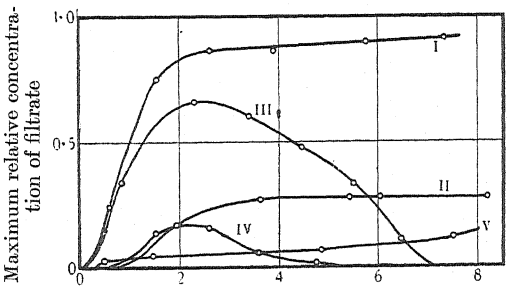

Fig. 1. Typical filtration curves.

filtrate, in terms of the concentration of protein in the stock solution (using the refractive index of the protein-free ultrafiltrate as a zero-point) could be expressed with an accuracy of about 2 %. It was plotted against the total volume filtered to give the "filtration curve" (Fig. 1).

The highest point on this curve, u_{max}, was characteristic of the solution and the membrane and was reproducible for comparable conditions to 5–10 %. The value of u_{max} is largely determined, in curves of type I, by the amount of adsorption of protein by the membrane; in curves of type II, by a process not fully explained, perhaps a true sieving mechanism; in curves of types III and IV, by the stage at which the narrowing and blocking of pores begins. These processes are discussed more fully below. In curves of type V, u is still rising at the end of the experiment, and u_{max} is significant only in comparing different filtrations which have all been carried to the same point.

Values of u_{max} for different membranes were plotted against the logarithm of membrane average pore diameter, log j, to give the "end-point curve" (Fig. 2). The end-point, j_e, for the disperse phase in a given system is the A.P.D.[1] of the membrane which just fully retains it.

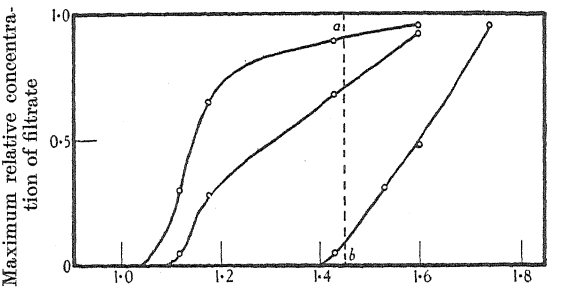

Fig. 2. Typical end-point curves.

The slopes of the end-point curves vary for different systems, as shown in Fig. 2. Hence the "filtrability" of the disperse phase in a given system cannot well be characterised by the end-point alone; it should be represented by the whole curve. We have selected three points on the curve for a convenient measure of filtrability: the values of average pore diameter, $j_{0·5}$, $j_{0·2}$ and j_e, which correspond with the maximum relative concentrations of filtrates 0·50, 0·20 and 0 respectively. The higher these values are, the less "filtrable" is the system.

The filtrabilities of different systems may be compared among themselves, however, by the values of u_{max} for one given grade of membrane, represented by the intersections of the end-point curves with the vertical line drawn through the appropriate value of log j, as shown in Fig. 2 by ab.

General effect of the nature of the solvent medium on filtrability.

The effect of capillary-active substances in decreasing the adsorption zone of the filtration curve and favouring filtration generally has been discussed [Elford, 1933]. The suspending medium uniformly used for filtration studies on viruses and bacteriophages in this Laboratory has been the Hartley's broth which is employed in bacteriological work. The stock broth is prepared from horse-flesh by extraction and digestion with trypsin [Hartley, 1922]. Its effect in facilitating filtration may be due to (1) peptisation of the particles of the disperse phase by adsorption of large residues of broken-down proteins; (2) lowering of surface tension of the solution; (3) peptisation and electrokinetic effects due to the presence of neutral salts.

[1] The abbreviation "A.P.D." is conveniently used for *average pore diameter*.

The stock broth often contains proteoses; addition of sulphosalicylic acid produces a precipitate which, in contrast with that given by the large serum-proteins, may be redissolved by boiling. Before investigating the influence of broth upon the filtrabilities of the serum-proteins, an effort was made to obtain a proteose-free broth. The standard method of preparation was modified by prolonging the digestion with 5 % Cole and Onslow's pancreatic extract for 2 weeks at 37°. Chloroform was added as a preservative. The resulting "extra-digested" broth gave a negative test with sulphosalicylic acid. Its surface tension at 21° was about 6 % lower than that of water, compared with a 25 % to 30 % lowering in the case of the stock broth. Its frothing power was greatly diminished.

Experimental.

Comparative experiments were made to show the effects of various media on filtrability. Solutions of serum-albumin, concentration 0·5 %, were made up in water, 1 % sodium chloride, and 1 : 1 dilutions in water of extra-digested broth, two different preparations of stock broth, and ox-heart broth (*i.e.* Hartley's broth prepared from ox-heart instead of horse-flesh). Each system, adjusted to approximately the same p_H, was filtered through a membrane of the same A.P.D. A similar experiment was performed with the pseudoglobulin, using 0·05 % sodium chloride solution instead of water. The results are shown in Table I.

Table I. *Filtration of serum-proteins in various solvent media.*

Concentration of protein 0·5 %.

Medium	Albumin. Membrane A.P.D. 14·5 mμ		Pseudoglobulin. Membrane A.P.D. 59 mμ	
	p_H	$u_{max.}$	p_H	$u_{max.}$
Water	7·1	0·14	—	—
0·05 % NaCl	—	—	7·1	0·14
1·0 % NaCl	6·9	0·34	7·3	0·61
Extra-digested broth	7·1	0·36	7·3	0·57
Stock broth, preparation 26–9	7·5	0·46	7·4	0·81
Stock broth, preparation 24–10	7·4	0·33	7·3	0·74
Ox-heart broth	7·4	0·46	7·4	0·73

Results. The data demonstrate the superiority of Hartley's broth as a solvent medium in the filtration of both proteins. Stock and ox-heart broths favour the filtration to about the same extent and are definitely more effective than extra-digested broth, which in turn has about the same effect as the 1 % sodium chloride solution. The superiority of the stock and ox-heart broths over the extra-digested preparation is probably due to the presence in the former of a capillary-active component, which becomes destroyed by prolonged digestion. It is also to be noted that in every instance the improvement in filtration is more marked for pseudoglobulin than for albumin. The mechanism of these effects will be discussed below.

Effect of p_H on filtrability.

Serum-albumin.

Experimental. The complete filtration and end-point curves were determined for filtrations of solutions of serum-albumin, concentration 0·4 % to 0·5 % in distilled water, at different values of p_H ranging from 3·0 to 8·8. The characterising

A.P.D. values, $j_{0\cdot5}$ and $j_{0\cdot2}$, were obtained by interpolation, and j_e by extrapolation on each curve. These three quantities are plotted against the p_H in Fig. 3.

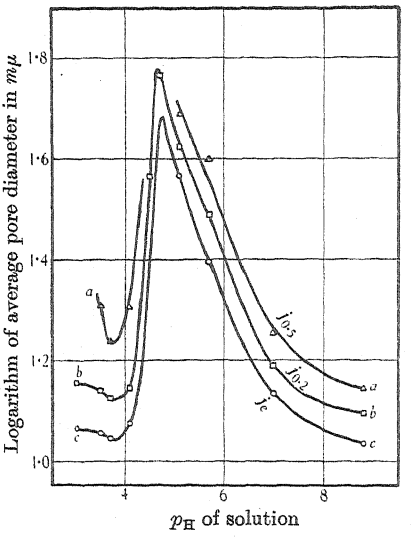

Fig. 3.

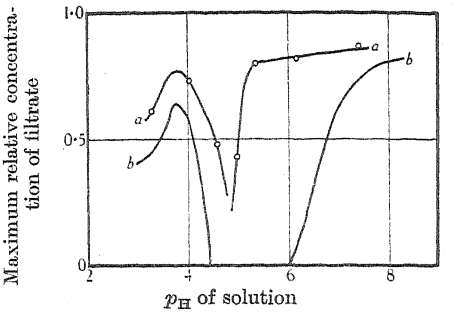

Fig. 4.

Fig. 3. p_H-filtrability curves of serum-albumin dissolved in water. *a*. $u_{max}=0\cdot50$. *b*. $u_{max}=0\cdot20$. *c*. $u_{max}=0$.

Fig. 4. Comparison of filtrability of serum-albumin dissolved in water and in Hartley's broth. *a*. Solution in Hartley's broth. *b*. Solution in water (interpolated from the data of Fig. 3). (Membrane A.P.D. $20\,m\mu$.)

Time did not permit a correspondingly complete series of end-point curves to be obtained for filtration of serum-albumin when dissolved in 1 : 1 dilutions in water of stock broth, but comparison was made of systems at different p_H values with one grade of membrane. The values of u_{max} are plotted against the p_H in Fig. 4, together with the corresponding curve for solutions in distilled water, interpolated from the data of Fig. 3.

Results. The high peak in Fig. 3, at which membranes of A.P.D. $45\,m\mu$ completely retain serum-albumin in aqueous solution corresponds fairly closely to its isoelectric point, p_H 4·88 [Svedberg, 1930]. It is significant that the adsorption of the protein on collodion particles as a function of the p_H exhibits a high maximum at the same point [Elford, 1933; Ettisch *et al.*, 1930]. Stock broth improves the filtration at all p_H values but most markedly at the isoelectric point and near it on the alkaline side. The minimum filtrability apparently occurs at the same p_H as in distilled water solution. At the optimum p_H values for filtration in aqueous solution, 3·8 and 8, the end-point is about $11\,m\mu$. The former p_H is just outside the zone within which Svedberg and Sjögren [1930] show serum-albumin to be monodisperse (p_H 4 to 9).

Serum pseudoglobulin.

Experimental. A complete set of end-point curves was established for filtrations of pseudoglobulin, concentration 0·4 %–0·5 % in 1 % saline, at different values of p_H ranging from 2·5 to 8. The values of $j_{0\cdot5}$, $j_{0\cdot2}$ and j_e, obtained as for

the albumin, are plotted against the p_H in Fig. 5. A comparison of filtrations in broth at different p_H values, similar to that performed for the albumin, is shown in Fig. 6.

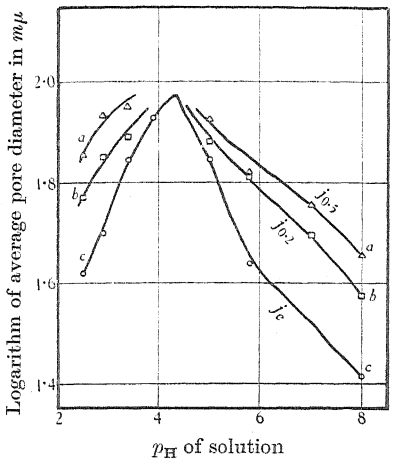

Fig. 5.

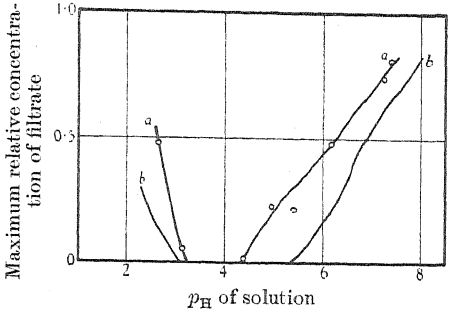

Fig. 6.

Fig. 5. p_H-filtrability curves of serum-pseudoglobulin dissolved in 1 % saline. *a*. $u_{max}=0·50$. *b*. $u_{max}=0·20$. *c*. $u_{max}=0$.

Fig. 6. Comparison of filtrability of serum-pseudoglobulin dissolved in 1 % saline and in Hartley's broth. *a*. Solution in Hartley's broth. *b*. Solution in 1 % saline (interpolated from the data of Fig. 5). (Membrane A.P.D. 59 $m\mu$.)

Results. Conditions for the poorest filtrability of pseudoglobulin in 1 % saline obtain at p_H 4·4, where a 90 $m\mu$ membrane completely retains the protein. This reaction is well on the acid side of the isoelectric point (p_H 5·4) in contrast to the close correspondence observed between isoelectric point and point of maximum j_e for the albumin. Adsorption of serum-globulin on collodion particles is greatest over a fairly wide range of p_H in the neighbourhood of the isoelectric point [Ettisch *et al.*, 1930; Elford, unpublished experiments], and the maximum is not sharply defined as in the case of serum-albumin.

Broth is found to improve the filtrability of pseudoglobulin at all p_H values (Fig. 6), but the point of minimum filtrability seems to be displaced still further in the acid direction (p_H 3·7). Since the broth itself exhibits a maximum opalescence at about p_H 3, the minimum filtrability observed at p_H 3·7 may depend upon the resultant stability of the broth colloids and of the pseudoglobulin.

The optimum point for the filtration of pseudoglobulin in 1 % saline, as far as the present investigations extend, is at p_H 8. The corresponding value for the end-point is about 25 $m\mu$.

Effect of neutral salt on filtrability.

Solutions of the albumin containing sodium chloride in concentrations varying from 0·05 % to 2·0 % were filtered at selected p_H values to show how increasing the amount of salt in the medium influenced filtration. The salt concentration producing the maximum effect in each case, together with the actual value of

u_{max}, are shown in Table II. The increase in filtrability due to the salt is least marked with systems of high filtrability (low j_e) in water, and the effect becomes greater as the isoelectric point is approached from the alkaline side.

Table II. *Effect of salt on the filtrability of serum-albumin.*

Concentration of protein 0·5 %.

p_H	Membrane $m\mu$	Concentration of NaCl %	u_{max} in NaCl solution	u_{max} in water	Δu_{max}	j_e in water $m\mu$
7·2	14·5	1·0*	0·34	0·14	0·20	13
6·8	20	0·8	0·90	0·63	0·27	14
4·8	40	2·0	0·95	0	0·95	45

* Only concentration used.

Effect of concentration of protein on filtrability.

Filtration studies on viruses have shown that the filtrability improves with increasing concentration of virus, particularly as the grade of membrane used approaches the end-point. The higher the virus concentration the more quickly is the adsorbing capacity of the membrane satisfied, so that the virus has the greater chance of appearing in the filtrate before blocking sets in. Therefore experiments have always been performed with suspensions of the highest concentrations obtainable, which, however, in the case of virus correspond to molecular concentrations which, for a protein, would be quite undetectable by the sulphosalicylic acid test. In protein solutions, the molecular concentration may be high enough actually to hinder filtration.

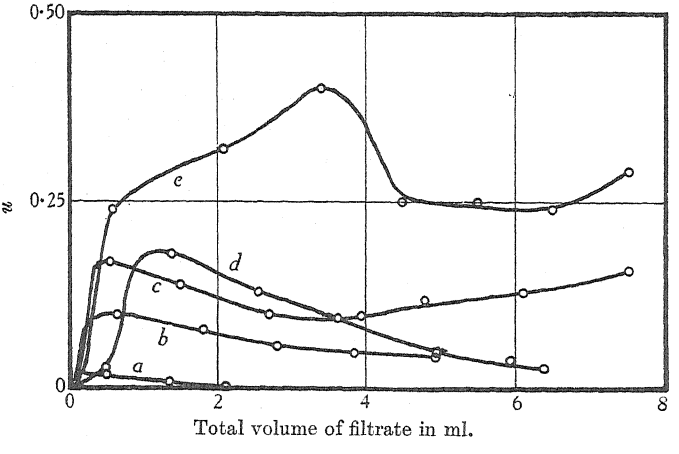

Fig. 7. Filtration curves of serum-albumin at different concentrations. *a*. 4·0 %. *b*. 2·0 %. *c*. 1·0 %. *d*. 0·5 %. *e*. 0·25 %. (Membrane A.P.D. 33$m\mu$; p_H of solution 5·7.)

Experimental. Solutions of serum-albumin at p_H 5·7–5·8 ranging in concentration from 0·25 % to 4 % were filtered through membranes of the same grade. The results are expressed in terms of the relative concentration of filtrate, u, in Fig. 7.

Results. The value of u_{max} decreases with increasing original concentration of the solution filtered. In the systems of highest protein concentration, the primary adsorption on the membrane is most quickly satisfied, but blocking sets in at such an early stage that the first sample of filtrate collected is the most concentrated. Under such conditions it is almost impossible to obtain reproducible results, as the filtrate concentration is rising so rapidly in the first ml. that a very slight variation of the time at which blocking becomes effective will result in widely conflicting values of u_{max}. Hence the concentrations of 4 %, 2 % and probably 1 % are unsuitable for quantitative comparative experiments. On the other hand, a concentration of 0·25 % is rather low for accurate analyses by the refractometer. The usual concentration used in these investigations, 0·5 %, is apparently the most satisfactory.

Effect of pressure on filtrability.

A solution of pseudoglobulin at p_H 5·2 was filtered through membranes of grade 84 $m\mu$ at pressures of 3, 2 and 1 atmospheres and under its own slight hydrostatic pressure in the filter. In the last case, the conditions approximated to those of a diffusion experiment. Fig. 8 demonstrates the effect of increasing

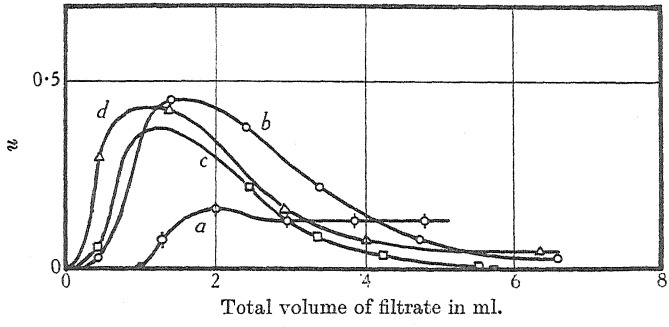

Fig. 8. Filtration curves of serum-pseudoglobulin at different pressures. *a*. No pressure applied. *b*. 1 atm. pressure. *c*. 2 atm. pressure. *d*. 3 atm. pressure. Membrane A.P.D. 84 $m\mu$; p_H of solution 5·2.

pressure in slightly narrowing the adsorption zone [Elford, 1933] and hastening blocking. The range of pressure (1–3 atmospheres) regularly employed in the present studies does not involve much variation in u_{max}.

Discussion.

The p_H-filtrability curves of serum-albumin (Fig. 3) indicate that the p_H range may be divided conveniently into characteristic zones of good and poor filtrability. Between p_H 4·2 and 7, there is a sharp peak of poor filtrability, where the end-point values of average pore diameter lie far above the molecular size (5·4 $m\mu$, disregarding the dissymmetry of the molecule) calculated from ultracentrifugation [Svedberg, 1930; von Mutzenbecher, 1933]. On each side this region grades into a zone of good filtrability, where the curves are relatively flat, and j_e is about twice the molecular size of the protein.

The filtration curves for experiments within the central zone of poor filtrability are invariably of types I, III and IV (Fig. 1). Curves of the other types occur

as this zone merges into the regions of good filtrability; the extreme acid and alkaline systems invariably give curves of types I, II and V. The universality of type I shows a uniform filtration mechanism for membranes which let through the respective systems in nearly undiminished concentration, *i.e.* where u_{max} lies between 0·9 and 1·0. The distribution of the other types indicates two different mechanisms in those filtrations where u_{max} lies between 0 and 0·9.

In a case where the p_H lies within the central zone, the relative concentration of the filtrate drops off at an early stage and decreases as the filtration is continued, eventually reaching zero. The pores have been rendered impermeable to protein, presumably blocked by large deposits of protein within them, and the u_{max} attained is far below the value which might have been reached had this blocking effect not occurred. This process may be termed *abnormal filtration*. On the other hand, when the experiment is made in the extreme acid or alkaline portion of the p_H range, the relative concentration of the filtrate reaches a definite fractional value which is fairly steadily maintained or may even rise gradually as the filtration continues. This is what would be expected for a true sieving mechanism, where the filtrate concentration slowly increases due to the continual gain in concentration of the residue above the membrane [Manegold, 1930] or is maintained constant if the latter effect is offset by a slow blocking. The suggestion of sieving need not necessarily mean that the system filtered is polydisperse. A monodisperse system filtered through a membrane whose pores are not quite uniform in size but have a range of size distribution would exhibit this effect; and our gradocol membranes, while believed to be more uniform than most other types of filters, possess such a range of pore sizes. But regardless of what interpretation is placed upon the filtration curves of types II and V, it is evident that only where these types obtain can the experiments be used for comparing the particle size of the disperse phase with the pore size of the membrane. Hence we propose to term the process which gives rise to these curves *normal filtration*.

The close resemblance in shape of the curve of j_e as a function of p_H for serum-albumin (Fig. 3) to the curve for adsorption of the protein on collodion particles [Elford, 1933] indicates the important correlation of adsorption with filtrability. However, the primary adsorption occurring at the pore surface, whose equilibration takes place within the first ml. or so of filtrate passing and is responsible for the initial steep rise in the filtration curve, is not the decisive phenomenon in filtration. It determines the value of u_{max} only in the case of curves of type I. The significance of the similarity of the curves is probably that the high peak in adsorption in the isoelectric region is due to the same process which causes the blocking of pores in abnormal filtration. That process may be a multiple layer adsorption.

The position and shape of the adsorption peak are the same whether the adsorbent is collodion, quartz, kieselguhr or kaolin (Elford, unpublished experiments), suggesting that the peak is due to adsorption not of protein on adsorbent only but of protein on protein, *i.e.* to the formation of secondary adsorbed layers. This corresponds with the tendency to aggregate which prevails in isoelectric protein solutions, as demonstrated by maximum opalescence and a minimum of the "alcohol number" defined by Fenn [1918] at this point. The blocking in the zone of abnormal filtration is then easily explained. When the protein system is forced through the membrane, multiple layers are adsorbed on the sides of the pores; the latter are narrowed and, as the filtration continues, eventually become blocked. The conditions within the pores are exceptionally favourable for formation of protein aggregates and precipitation, owing to the frequency of

collisions in the limited space. On the other hand, in the zones of normal filtration (which may be termed the stable[1] zones), adsorption of the protein is probably unimolecular. The pores are lined with one layer of protein, and other protein molecules slip through without being retained.

The range of p_H values investigated for pseudoglobulin seems to lie entirely within its abnormal filtration zone (Fig. 5), as the end-point at the extreme alkaline side is still more than three times the size of the molecule (6·9$m\mu$, disregarding molecular dissymmetry). This is confirmed by the filtration curves, which are nearly all of the types I, III and IV. The broad zone of abnormal filtration is quite in agreement with the fact that solutions of pseudoglobulin are generally less stable and more apt to aggregate and precipitate than those of albumin. In solutions whose p_H values lie in the centre of this zone, even the adsorption on the top surface of the membrane apparently suffices to initiate a progressive precipitation. The residue above the membrane after filtration of such a system usually contains a copious precipitate of protein, and the concentration of the supernatant solution, in spite of a decrease in volume of ten- or twenty-fold, is less than that of the stock solution originally introduced into the filter. The adsorption-p_H curve of the pseudoglobulin, again, closely resembles the filtrability curves.

The general effect of salt in improving filtrability, observed at the isoelectric point and on the alkaline side of it, may now be interpreted as a peptising action due perhaps to alteration in charge and hydration of the protein molecule by adsorption of ions. It suppresses aggregation and formation of multiple adsorbed layers and renders filtration less "abnormal." The more abnormal the filtration is in the absence of salt, the more the presence of salt would be expected to affect it; and this is found to be the case (Table II). This is further illustrated by the fact that saline medium improves the filtrability of pseudoglobulin more than that of albumin, solutions of the former protein being characterised by their relative instability and abnormal filtration. The stabilising effect of salt on the globulin is also apparent in decreasing visible opalescence and increasing the "alcohol number." An increase in the electrolyte content of the system will also progressively reduce the Donnan potential at the surface of the protein particle and thus tend to minimise any consequent electro-viscous effects.

The extra-digested broth probably peptises the protein in much the same way as does the sodium chloride, owing to its high content of salts and amino-acids (Table I). The other broths, however, have an additional effect, and this is probably "lubrication" due to preferential adsorption of a capillary-active constituent on the surface of the membrane pores [Elford, 1933], and consequent suppression, not only of multiple-layer adsorption, but even of unimolecular adsorption of the protein, thereby making conditions the most favourable possible for filtration.

Stock broth can thus improve the filtrability of albumin even at a p_H at which filtration in distilled water solution is normal. Fig. 4 demonstrates that the filtrability is, in fact, increased by broth over the entire p_H range, and the zone of abnormal filtration is much narrower (p_H 4·3–5·5) than for aqueous solutions of the protein (p_H 4·2–7).

[1] Our use of this term should not be confused with that of Svedberg, whose "p_H-stability ranges" refer to stability of monodispersion, with respect to breakdown (occasionally aggregation) of protein molecules. "Stability" is used in this paper with respect to potential aggregation and precipitation.

Optimum conditions for filtration.

It is essential in determining the filtration "end-point" for estimating the particle size of a disperse system to ascertain and utilise the optimum conditions for filtration. These obtain when the filtration is "normal," and when the end-point value is as close to the actual particle size as possible. The fact that a protein in solution, as found by Svedberg in his ultracentrifugation studies, is uniformly monodisperse only within certain definite characteristic ranges of p_H, makes it necessary to confine filtration within these prescribed limits. In the studies with virus suspensions also, a restriction upon the permissible working conditions is imposed by the p_H range of viability in each instance. The evidence presented in the foregoing sections clearly indicates that for the serum-proteins the requirements for optimum filtration conditions would be fulfilled by adopting Hartley's broth, at p_H 8, as the standard suspension medium. This medium at p_H 7·4–7·6 has been consistently used in ultrafiltration analyses of virus and bacteriophage suspensions in this laboratory. Experience has shown that both virus and phages filter under these conditions much more readily than when contained in saline or Ringer solution [Galloway and Elford, 1931; Elford and Andrewes, 1932]. Further, their filtrability in broth is not appreciably affected by changes of p_H within the range of viability whereas in saline and Ringer the filtrability of the viruses and phages decreases as the p_H is changed in the acid direction. It must be remembered in this connection, however, that when working with viruses it is seldom possible to detect with certainty variations in concentration less than tenfold.

The ratio, particle size/end-point A.P.D., for the serum-proteins.

An empirical correction factor, derived from filtration studies with suspensions of known particle size, has been used in calculating the particle sizes of viruses and bacteriophages from the values of their experimentally determined end-points [Elford, 1933]. The ratio, particle size/end-point A.P.D., was evaluated individually for certain bacteria, gold sols, haemoglobin and egg-albumin. The correction factor was given as ranging from this ratio to a figure about 30 % below it. It is of interest to derive the values of the ratio for the serum-proteins (Table III). The sizes of the protein particles, denoted by $2r$, are calculated from

Table III. *Particle sizes and end-points of the serum-proteins.*

	Medium	Particle size, $2r$ $m\mu$	End-point j_e ($m\mu$)	Ratio $\frac{2r}{j_e}$
Serum-albumin	Water, p_H 8·0	5·4	11	0·49
Serum-albumin	Broth, p_H 7·8	5·4	9–10	0·54–0·60
Serum-pseudoglobulin	Broth, p_H 7·8	6·9	11–12	0·57–0·63

Svedberg's and von Mutzenbecher's values for the molecular weights, neglecting their slight dissymmetry and regarding them as spherical. The end-points under the optimum conditions, *i.e.* solution in Hartley's broth at p_H 7·8, were obtained by extrapolation from the lower portions of the end-point curves of the respective systems. The end-point for serum-albumin in water at p_H 8·0 is from the data of Fig. 3.

The end-point for the albumin in water is naturally higher than in broth, even though the filtration is quite normal in both cases, because of the additional "lubrication" afforded by the latter medium. The ratios $\frac{2r}{j_e}$ for the two proteins

in broth agree satisfactorily with the value (0·54) for haemoglobin in the same medium [Elford, 1933].

It is hoped to present later similar data for proteins of still larger molecules, when a general tabulation of the values of the ratios will be made.

Filtration of artificial mixtures.

Examination of the respective end-point curves showed that at p_H 3·8 membranes of A.P.D. 40 $m\mu$ to 80 $m\mu$ retain pseudoglobulin (in saline) completely, whereas albumin (in distilled water) passes them in nearly undiminished concentration. To determine the behaviour of a mixture of the two, a solution at p_H 3·8 was made up to 0·5 % in respect to each protein in 1 % saline and filtered through several grades of membranes in this range. The results are shown in Table IV.

Table IV. *Filtration of artificial mixtures.*

0·5 % albumin + 0·5 % pseudoglobulin in 1 % NaCl p_H 3·8.

Membrane A.P.D. $m\mu$	Pseudoglobulin (1/2 sat. $(NH_4)_2SO_4$)	Albumin (sulphosalicylic acid)
70	Positive	Positive
55	Trace	Trace
40	Negative	Negative

Under these conditions, the filtrability of the pseudoglobulin is increased, and that of the albumin is greatly decreased. The improvement in the case of the former protein is probably the result of a stabilising influence exerted by the albumin. On the other hand, the drop in filtrability of the albumin no doubt means that the pores, clogged by multiple adsorbed layers of pseudoglobulin (here undergoing abnormal filtration), are blocked to both proteins equally. Hence the data do not necessarily indicate formation of an albumin-globulin complex. (The mixture is not strictly comparable with the pure solution of albumin, owing to the presence of the sodium chloride in the former; but any effect due to the salt would be small compared with the great decrease in filtrability observed).

Filtration of horse serum.

In order to compare the filtration end-points of serum-albumin and globulin as determined in pure solutions and in their native state in serum, reference is made to the work of Elford, Grabar and Fischer. Their experiments, details of which will be published later, yield the end-point values for the proteins in undiluted serum and in dilutions in broth and in saline (Table V).

Table V. *End-points of the serum-proteins in native serum.*

	Pressure 1–3 atm. p_H	End-point of albumin $m\mu$	End-point of globulin $m\mu$
Undiluted serum	8·2	9–10	11
Serum: broth, 1 : 9	7·6–7·8	9–10	11
Serum: saline, 1 : 9	7·8–8·0	11	13

It is seen by inspection of Tables III and V that the filtrability of each serum-protein is characterised by the same end-point whether the system filtered is normal serum or the pure solution of the protein in Hartley's broth at p_H 7·8.

This indicates that serum-albumin and globulin exist largely as individual molecular species in their native state in serum, a conclusion also reached by Svedberg and von Mutzenbecher in their ultracentrifugation studies.

Summary.

1. The filtration curve, end-point curve and a quantitative measure of filtrability are defined.

2. The influence of the nature of solvent medium, p_H, neutral salts, concentration of protein and filtration pressure on the ultrafiltration of serum-albumin and pseudoglobulin through graded collodion membranes is described.

3. These effects demonstrate two types of filtration, termed normal and abnormal, and are interpreted in terms of adsorption of protein within the membrane pores.

4. Hartley's broth at p_H 7·6–7·8 is found to be the best solvent medium for filtration experiments with the proteins, as previously noted for bacteriophages and animal viruses.

5. The ratios, particle size/end-point A.P.D., are given for the two proteins for optimum filtration conditions.

6. The filtration end-point of each protein is the same in pure solution in Hartley's broth at p_H 7·8 as in native horse serum.

REFERENCES.

Barnard and Elford (1931). *Proc. Roy. Soc. Lond.* B **109**, 360.
Burnet and Andrewes (1933). *Zentr. Bakt. Par.* Abt. 1, **130**, 161.
Elford (1931). *J. Path. Bact.* **37**, 505.
—— (1933). *Proc. Roy. Soc. Lond.* B **112**, 384.
—— and Andrewes (1932). *Brit. J. Exp. Path.* **13**, 446.
—— and Ferry (1934). (To be published shortly.)
Ettisch, Domontowitsch and von Mutzenbecher (1930). *Naturwiss.* **18**, 447.
Fenn (1918). *J. Biol. Chem.* **33**, 279.
Galloway and Elford (1931). *Brit. J. Exp. Path.* **12**, 407.
Hartley (1922). *J. Path. Bact.* **25**, 479.
Hopkins and Pinkus (1898). *J. Physiol.* **23**, 130.
Manegold (1930). *Kolloid Z.* **51**, 220.
von Mutzenbecher (1933). *Biochem. Z.* **266**, 226, 250.
Svedberg (1930). *Trans. Far. Soc.* **26**, 737, 740.
Svedberg and Sjögren (1930). *J. Amer. Chem. Soc.* **52**, 2855.

XCI. THE REVERSIBLE ENZYMIC OXIDATION OF VITAMIN C.

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Some years ago during the progress of an inquiry on the production of an antiscorbutically active cider from Bramley's Seedling apples it was observed that the juice expressed from the fruit while retaining a very high proportion of its antiscorbutic activity did not reduce indophenol. At that time the writer held the view that the capacity for reducing this indicator was mainly due to a substance which, although closely associated with the vitamin in a protective way, was not the active principle itself. The probability of the production of a reversibly oxidised active form of the vitamin was not at that time seriously considered by him.

This problem was reinvestigated recently in view of the fact which has now been established that *l*-ascorbic acid possesses antiscorbutic activity *per se* and that when this compound is reversibly oxidised [Szent-Györgyi, 1928] it retains its activity [Tillmans *et al.*, 1932; Hirst and Zilva, 1933].

It was found again that juice expressed from Bramley's Seedling apples although unable to reduce indophenol was antiscorbutically active. Thus a daily dose of 3 ml. of the non-reducing apple juice afforded protection to guinea-pigs equivalent to 0·25–0·5 mg. of ascorbic acid or to rather less than 1 ml. of decitrated lemon juice (10 I.U.). This potency is not very much less than that obtained with intact tissue of these apples (usually about 15 I.U. per 3 g.). The apple juice, it was further found, reduced indophenol immediately after expression but rapidly lost this power of reduction. Aqueous extracts prepared with or without cyanide, unlike alcoholic, trichloroacetic acid and strongly acid extracts did not reduce indophenol. Furthermore, the non-reducing apple juice after treatment with hydrogen sulphide regained the property of reducing indophenol. This evidence is very suggestive of a case of enzymic reversible oxidation. Experiments will here be described which strongly support this hypothesis.

Experimental.

The oxidation of vitamin C in lemon juice.

15 ml. of freshly expressed apple juice (Bramley's Seedling), which did not reduce indophenol, were added (*a*) to 50 ml. of decitrated lemon juice p_H 7 (resulting p_H of mixture, 4·4); (*b*) to 50 ml. of decitrated lemon and the mixture adjusted to p_H 3 (reaction of apple juice). These mixtures were allowed to remain in the presence of air at room temperature for 6 hours. At the end of this time the reduction in (*a*) had fallen from 4·9 to 0·8 ml. *N*/1000 indophenol per ml.; in (*b*) from 4·9 to 2·1 ml. of the indicator. The corresponding control solutions of decitrated lemon juice which did not contain any apple juice lost very little of

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their indophenol reducing power. The oxidised solutions were then submitted to reduction with hydrogen sulphide as described by Johnson [1933]. The titre of (*a*) and of (*b*) rose in consequence to 6 ml. *N*/1000 indophenol. These figures were higher than the original (4·9 ml.) owing to the fact that the oxidised apple juice in the samples was also reduced in the process. An experiment performed with pure ascorbic acid in aqueous solution gave similar results.

The biological activity of the reversibly oxidised vitamin C in lemon juice.

Decitrated lemon juice treated daily as above and kept at p_H 4·4 was used in these tests. The indophenol reducing power left in these preparations after they had stood for 6 hours varied with the samples of the apple used. Only very seldom was the reducing power found to have disappeared entirely. Occasionally oxidation was not very advanced after this time. In such cases the preparation was allowed to remain for about 24 hours, after which no reducing power could usually be observed. It was, however, considered inadvisable to test 24-hour samples daily in order to avoid the possibility of considerable direct oxidation by atmospheric oxygen. Daily doses of 1·5 ml. of the oxidised preparations were tested, and decitrated lemon juice of an equivalent reducing capacity was administered to a control group of guinea-pigs. It will be seen from Fig. 1 that the

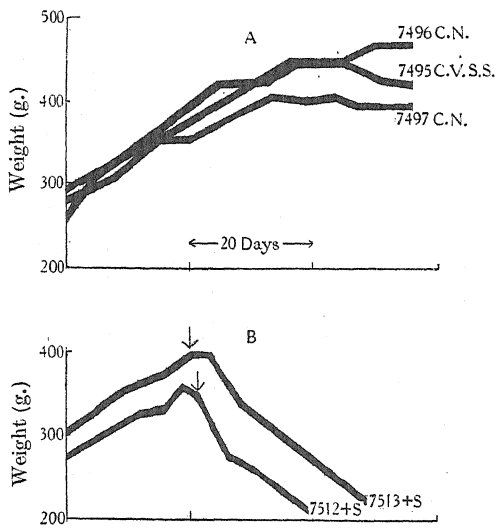

Fig. 1.

C = Chloroformed. V.S.S. = Very slight scurvy.
+ = Died. N = Normal.
S = Scurvy. ↓ = Onset of clinical symptoms of scurvy.

A. These animals received 1·5 ml. of reversibly oxidised decitrated lemon juice daily. Average reduction value of daily dose 1·4 ml. *N*/1000 indophenol.

B. These animals received as a control untreated decitrated lemon juice. Average reduction value of daily dose 1·8 ml. *N*/1000 indophenol.

reversibly oxidised dose of 1·5 ml. which showed an average daily reducing capacity of only 1·4 ml. *N*/1000 indophenol protected the animals to a very marked extent, whilst a dose of decitrated lemon juice of a similar reducing

capacity afforded hardly any protection. As the activity of apple juice present in the oxidised dose was negligible it may be assumed that the reversibly oxidised vitamin of the decitrated lemon juice was mainly responsible for the protection.

The influence of the absence of oxygen on the action of apple juice on vitamin C.

Experiments similar to the above were carried out at p_H 4·4 in an atmosphere of nitrogen. In a typical experiment the solutions were placed in ampoules which were then alternately exhausted and filled with nitrogen three times. These solutions as well as the controls, which were kept in conical flasks in the air, were stored side by side at room temperature for 24 hours. While the reducing power of the control solution fell from 5·5 ml. N/1000 indophenol to nil per ml. that of the anaerobic sample fell only to 4 ml. N/1000 indophenol. The small loss in reducing power in the latter was most probably due to manipulation, and reversible oxidation, if any, was therefore small.

The influence of boiled apple juice on vitamin C.

Experiments similar to the above were performed in which the apple juice used was previously boiled. The following is a typical experiment in which the reducing capacity of the sample containing the boiled apple juice fell at p_H 4·4 from 4·9 to 3·8 ml. N/1000 indophenol per ml. of solution in 6 hours, a fall which was very probably due to atmospheric oxidation. In the sample with the unboiled juice the original titre fell to 0·8 ml. N/1000 indophenol per 1 ml. of solution.

The influence of boiled apple juice with added peroxidase on vitamin C.

The apple juice used in the above experiments is capable of oxidising the usual peroxidase reagents in the presence of hydrogen peroxide. This oxidative activity is destroyed on boiling, and it was of interest to discover whether the addition of peroxidase to boiled apple juice would restore its capacity to oxidise vitamin C. The preceding experiment was therefore repeated with and without the addition of turnip-peroxidase to the boiled apple juice. It will be seen from the following representative experiment that this addition did not restore the oxidising activity of the boiled apple juice. Thus at p_H 4·4 the solution containing the boiled apple juice alone and the solution containing the boiled apple juice *plus* a few drops of turnip-peroxidase solution, as well as the control decitrated lemon juice containing no apple juice, fell in their reducing capacity from 5·1 to 4·5 ml. N/1000 indophenol per ml. of the solution.

The influence of cyanide on the action of apple juice on vitamin C.

As cyanide inhibits peroxidase activity, an endeavour was made to ascertain whether this reagent would also inhibit the oxidising activity of the apple juice. In these experiments carried out at p_H 4·4 the usual quantities of decitrated lemon juice and apple juice were used. To one sample sodium cyanide was added to make the concentration M/500. This concentration inhibited the peroxidase activity in the solution. The following is a typical example. In the sample containing cyanide the indophenol reducing capacity fell from 5·3 to 1·8 ml. N/1000 indophenol per ml. in 6 hours. In the control sample which did not contain any cyanide the reducing capacity fell to 1·1 ml. in the same time. This concentration of cyanide, therefore, had no serious inhibiting effect on the oxidation of the vitamin. Only when the concentration was raised ten- or twenty-fold was a marked inhibition observed.

Discussion.

The experiments described supply fairly convincing evidence that the apple contains an enzyme which is capable of reversibly oxidising vitamin C without affecting its antiscorbutic potency to any very marked extent. The facts that peroxidase when added to boiled apple juice is incapable of oxidising the vitamin and that quantities of cyanide which inhibit this enzyme do not seriously interfere with the oxidation of the reduced form of the active principle, show that this oxidation is not brought about by peroxidase either alone or as one of the enzymes in a coupled system. The oxidising enzyme seems to resemble the one described by Szent-Györgyi [1931].

This worker found that unboiled, but not boiled, cabbage pulp rapidly lost its capacity for reducing Folin's phenol reagent and for taking up oxygen in a respirometer. Further, the addition of hexuronic acid (ascorbic acid) to cabbage juice greatly increased the uptake of oxygen. He concluded that cabbage contained an enzyme (hexoxidase) which could oxidise reversibly hexuronic acid. The oxidised product obtained by him was not tested for its antiscorbutic activity, as at that time the identity of ascorbic acid with vitamin C was not known. It is, however, quite conceivable that the oxidised product in cabbage juice is also active.

That the oxidising enzyme functions under physiologically controlled conditions in the tissue of the intact apple as well as in the disrupted fruit is more than probable. In fact, evidence which is very suggestive in this respect has been obtained during the last few years in an enquiry on the vitamin C of the apple by the writer in collaboration with Drs F. Kidd and C. West of the Low Temperature Research Station, Cambridge. The knowledge of the distribution of this enzyme in the plant and animal kingdoms would evidently greatly contribute to the solution of the problem of the function of vitamin C in plant and animal organisms.

Summary.

The apple (Bramley's Seedling) contains a thermolabile enzyme which is capable of reversibly oxidising vitamin C without seriously impairing its antiscorbutic activity.

This enzyme does not function under anaerobic conditions and is only inhibited by very high concentrations of cyanide.

The peroxidase of the apple does not seem to be involved in this oxidation process.

REFERENCES.

Hirst and Zilva (1933). *Biochem. J.* **27**, 1271.
Johnson (1933). *Biochem. J.* **27**, 1287.
Szent-Györgyi (1928). *Biochem. J.* **22**, 1287.
—— (1931). *J. Biol. Chem.* **90**, 385.
Tillmans, Hirsch and Dick (1932). *Z. Unters. Lebensm.* **63**, 267.
—— —— and Siebert (1932). *Z. Unters. Lebensm.* **63**, 21.

XCII. THE FORMALDEHYDE-AZO-TEST FOR VITAMIN B_1.

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THERE is some vagueness as to the exact scope of the so-called Pauly reaction, such as is given by histidine with diazotised sulphanilic acid. In the broad sense, it might be considered to characterise any substance reacting in a weak alkaline solution to give the characteristic pink or red colour. In the particular sense however it is often now taken to indicate a substance reacting with the reagents studied in detail by Koessler and Hanke [1919; see also Hunter, 1922; 1925].

Jansen and Donath [1926] stated that their crystalline preparations of vitamin B_1 from rice polishings gave a strong Pauly reaction, *i.e.* gave a red colour when treated with diazotised sulphanilic acid in presence of sodium carbonate. On the other hand, Kinnersley and Peters [1927; 1928] found (*a*) that most of the substances in yeast concentrates of vitamin B_1 giving the Pauly reaction (Koessler and Hanke technique) did not fractionate with the activity and (*b*) that fractions of activity 10γ per unit of B_1 did not contain more than 3 % of substances giving a Pauly reaction similar to that of histidine. Guha and Drummond [1929] also obtained active vitamin B_1 fractions from yeast giving feeble Pauly reactions. In ignorance of the true potency of vitamin B_1 itself, it was impossible to decide whether the final entity was responsible for the trace of pink found. In 1931, at the British Association, it was stated [Peters, 1931] that the trace of pink colour given in the Pauly reaction by active yeast concentrates and by Jansen's crystals could be enhanced by suitable choice of alkalinity, and that it was believed that the active entity gave a Pauly reaction[1]. Shortly afterwards this opinion was confirmed in further work, the outcome of which is given in this paper. Meanwhile other researches have given contradictory results, *e.g.* the vitamin B_1 crystals of Ohdake [1932] are stated to give a slow pink diazo-reaction, whereas the crystalline preparations of Tschesche *et al.* [1931] and of Van Veen [1932] gave no Pauly reaction, though the latter author was of the opinion that interaction with diazotised sulphanilic acid took place. Our own crystalline preparations have been stated by us to give an entirely yellow reaction with Pauly's test as performed by Koessler and Hanke, *i.e.* in 2·5 % sodium carbonate. Hence we were correct in attributing our previous 3 % to impurities.

Nevertheless, in essence the opinion as expressed at the British Association Meeting is correct, namely that the vitamin would interact with the diazo-reagent to give a pink colour at a more alkaline reaction than the one employed in the Koessler and Hanke method. We have delayed publication on this question for over two years in the effort to collect conclusive evidence, and we are now presenting shortly some of our data (as a preliminary step). Two circumstances have assisted our progress: (1) the realisation that rather fine shades of alkalinity

[1] It should have been added "in this sense."

would influence the coupling of some substances in the yeast extracts and the degree of yellow rather than pink formed (it is well known that pronounced changes in the alkalinity influence these colours): (2) the discovery of the remarkable effect of formaldehyde.

Test in presence of formaldehyde.

All vitamin B_1 crystalline preparations in our possession interact to produce a pink colour with the following reagent:

N NaOH	100 ml.
$NaHCO_3$	5·76 g.
H_2O	100 ml.

Method of performing test. 0·5 ml. of the diazotised sulphanilic acid[1] (Koessler and Hanke) is added to 1·25 ml. of the reagent in a small test-tube and after 1 minute 1 drop (0·03 ml.) 40 % formaldehyde and then immediately the vitamin in 0·1–0·3 ml. solution of acidity greater than p_H 4·0. A pink colour develops slowly and increases in intensity for 30–60 minutes. After this time it remains approximately constant, though the pink becomes more prominent up to 24 hours owing to fading of the yellow. Standards can be made up with different concentrations of the vitamin from 2 to 6 or 7 pigeon doses and kept for about 14 days. The limit of sensitivity is about 1 vitamin B_1 unit, approximately 1·5–2·0γ crystals. If made up in steps of 1 unit (pigeon dose) in small test-tubes (7 × 1·3 cm.) and corked, reasonably accurate estimates of colour can be made with 5–10γ of vitamin crystals. Trials with a colorimeter have not given as satisfactory results with these dilute solutions as the simple comparative method. The latter can be combined with dilution if desired. With amounts of 1 unit, the colour is ill-defined. Even in the purest specimens of vitamin yet reached, the red is always tinged with yellow. 10γ (5 pigeon doses in our sense) approximately match a mixture of Sørensen's acid and alkaline phosphates (2+8 volumes) of p_H 5 at 15°, containing in 103 ml., 100 ml. of phosphate mixture and 3·0 ml. 0·02 % phenol red.

Influence of formaldehyde.

The N/10 HCl extracts from charcoal [Kinnersley *et al.* 1933]. In the purer preparations of 5γ and under per vitamin B_1 unit, the formaldehyde stabilises the colour. In its absence the colour increases in intensity for a short time and then fades. This perhaps explains Ohdake's [1932] observations. The pink-coloured substance is extracted by butyl alcohol from alkaline solution, and so can often be separated from the yellow substances in impure solutions.

In the less pure preparations up to 100γ per unit the formaldehyde has the effect of suppressing or inducing the gradual fading of the yellow colour caused by impurities (such as histamine), which react with the reagent at the particular alkalinity chosen to give a yellow colour. (At a less alkaline p_H such substances would of course give the usual pink.)

The 50 % alcohol extracts from charcoal and baryta extract of Jansen's acid clay standard. In these cases increase of the formaldehyde to a point at which the vitamin red colour is suppressed is not sufficient to inhibit the yellow colour. Orange colorations are therefore always produced. No attempt has yet been made to explore this further. By use of the Rosenheim-Schuster colorimeter, the red component can be selected and could probably be estimated, but this has not been properly explored.

[1] This reagent will keep for a week, if surrounded by ice in a thermos flask.

In all the impure extracts there is apt to be present in addition a substance giving a colour reaction at a more alkaline p_H. The alkalinity is chosen to reduce to a minimum the influence (*a*) of substances of the histamine type showing pink at more acid reaction and (*b*) of substances showing colours at a more alkaline reaction.

Vitamin B_1 couples at an acid as well as an alkaline reaction. The azo-colour is only produced at an alkaline p_H. The test cannot well be applied at all to extracts less pure than 500γ/unit, as it is obscured by other reactions. In yeast extracts (and other sources investigated), there may be present at the earlier stages (in the extracts of acid charcoal for instance) a substance behaving similarly to the vitamin. It may be distinguished by adding to the pink solution concentrated HCl drop by drop to approximately p_H 8. At this p_H, the colour in question rapidly diminishes, whereas that of the vitamin persists for some time, even at an acid p_H. Beyond this, the test appears to have a fairly high degree of specificity; out of a large number of substances tried, one of the few giving the reaction is acetone, and the colour in this case is not permanent. The question however has not been thoroughly explored. The formaldehyde should be added to an acid solution of the vitamin immediately before the diazotised reagent. An explanation of the action of formaldehyde is not to hand; possibly it is in this case an oxidising action. It has been noticed that methylene blue reduced by sodium hydrosulphite at an acid p_H is not affected by addition of formaldehyde; making alkaline, however, induces much more rapid oxidation of the methylene white in presence than in absence of formaldehyde.

A preliminary exploration with this test has been made of a large number of extracts of seeds, roots, sugars, *etc.*, after treatment with lead acetate and charcoal for the extraction of the vitamin. In many cases there was a substance producing the vitamin type of colour unstable in acid; in no case was there reason to suspect a more active source of the vitamin than yeast. It should be emphasised that in first extracts the concentration of the vitamin is never sufficient to show the reaction.

Table I shows a comparison between vitamin activities by bird and formaldehyde-azo-test; it represents a few only of the numerous estimations made.

Table I.

Amounts (γ) having similar activities (=4 pigeon day doses).

(1) Older preparations

No.	Bird γ	Formaldehyde-azo-test γ
53	10	10
Windaus *et al.*	17·8	18
64·19	10	9·6
56	11·2	13·8

(2) More recent tests

No.	Bird γ	Formaldehyde-azo-test γ
92 A 50	8	8
19	10 (5)*	8·9
Van Veen	8 (10)†	6·8
93 A	10 (7)	12·8

(3) Tests during an early fractionation of a phosphotungstate fraction, p_H 5·5–4·8. p_H refers to the p_H at which fractions were separated.

p_H	Bird (γ)	Formaldehyde-azo-test (γ)
5·3+	51·5 (5)	51·5
5·2+	125 (4)	120
5·0−	157 (4)	230

* Figures in brackets refer to numbers of birds in tests. For probable errors see paper by Kinnersley *et al.* [1933], and for comparison with catatorulin tests Passmore *et al.* [1933].

Note. 1 pigeon day dose = approximately 1 vitamin B_1 unit.

† This is not the preparation recently mentioned in *Nature*.

Tests upon the preparations of Tschesche *et al.*, and Van Veen[1] are also included. The latter is from rice polishings, so that the reaction is given by oryzanin as well as by torulin.

There is no question from an examination of Table I of the close parallelism between the fractionation of vitamin B_1 and the substance in the extract giving the formaldehyde-azo-test. We are clear, however, that we have had in our hands occasionally forms of the vitamin which do not give the test at the same intensity as the bird test. Hence, though it can be said that with the above exception all substances giving this test in these extracts contain vitamin B_1, it is apparently not true that all forms of vitamin B_1 give the test.

Use of the test in fractionation.

The formaldehyde-azo-test is of the greatest value in the fractionation and has enabled us almost to dispense with the animal tests in fractionation of the phosphotungstate extract from the $N/10$ HCl extracts of the charcoal [Kinnersley *et al.* 1933]. By carrying out simultaneous tests with and without formaldehyde during the fractionation at p_H 4, it is possible to separate almost exactly all the vitamin from the substance which gives the yellow reaction, mostly destroyed by formaldehyde under our conditions[2]. Until it is quite certain that pure vitamin B_1 has been obtained, it cannot be proved that the formaldehyde-azo-test is one of its properties. But it can be definitely asserted that the test is of the greatest value in following vitamin B_1 up to a highly potent crystalline stage. This assertion is based upon a large number of experiments over a period of two years. It is possible that the discrepancies still shown in Table I are not due to experimental error (*e.g.* 93A); this should be decided by work in progress in collaboration with J. R. O'Brien.

SUMMARY.

An azo-test is described of high specificity for vitamin B_1. The specificity is due to the suitable choice of alkalinity for coupling and the use of formaldehyde as a stabilising agent.

We are indebted to the Medical Research Council for a personal grant to one of us. We are also grateful to Mr J. R. O'Brien for advice and help.

REFERENCES.

Guha and Drummond (1929). *Biochem. J.* **23**, 880.
Hunter (1922). *Biochem. J.* **16**, 640.
—— (1925). *Biochem. J.* **19**, 25.
Jansen and Donath (1926). *Proc. K. Akad. Wetensch. Amsterdam*, **29**, 1390.
Kinnersley and Peters (1927). *Biochem. J.* **21**, 789.
—— —— (1928). *Biochem. J.* **22**, 422.
—— O'Brien and Peters (1933). *Biochem. J.* **27**, 232.
Koessler and Hanke (1919). *J. Biol. Chem.* **39**, 505.
Ohdake (1932). *Bull. Agric. Chem. Soc. Japan*, **8**, 111.
Passmore, Peters and Sinclair (1933). *Biochem. J.* **27**, 542.
Peters (1931). *Chem. Brit. Assoc.* 141. (Heffer.)
Tschesche, Ruhkoff, Laqueur and Schultz (1931). *Nachricht. Ges. Wissenschaften Gött.* 207.
Van Veen (1932). *Rec. Trav. Chim. Pays-Bas*, **51**, 279.

[1] We again thank Dr Tschesche and Dr Van Veen for the gift of these preparations.

[2] Without formaldehyde, the pink colour gradually fades.

XCIII. THE ACTION OF GASTRIC JUICE ON BEEF MUSCLE-GLOBULIN, WITH REFERENCE TO ANAEMIA.

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Pernicious anaemia results from a failure of the supply of healthy red cells to the circulating blood. No way was known of restoring this lost power of regeneration of healthy red cells until Minot and Murphy [1926] observed that by feeding liver to persons suffering from this type of anaemia increased numbers of reticulocytes, or young red blood cells, appeared in the circulation, with the result that the blood was gradually restored to its normal condition.

Following on this discovery, Castle [1929; Castle and Townsend, 1929] announced that changes in the blood, similar to those produced by liver feeding, could be caused by daily administration of raw beef which had previously been digested by normal gastric juice, either in the stomach of a normal person or *in vitro*. Neither beef nor gastric juice was effective alone or when fed successively. Castle suggested that pernicious anaemia is a deficiency disease of a new type; that it is due to a lack of some unknown substance or principle produced from protein during normal digestion; and that the gastric secretion in pernicious anaemia is deficient in a factor necessary for the production of this principle.

According to this work there are, therefore, two factors involved in the maintenance of the proper supply of red blood cells: (1) an intrinsic factor in the gastric juice, and (2) an extrinsic factor contained in protein. By the interaction of these factors the haematopoietic principle is produced. Normally this principle is concentrated in the liver, hence the efficacy of this organ in the treatment of anaemia.

In his later work Castle *et al.* [1930] demonstrated that the protein of beef muscle, precipitated at p_H 6 and washed with saline of the same reaction, could act as the extrinsic factor: washed caseinogen and wheat gluten were unsuitable. The principle could be obtained by digesting this precipitated beef protein with normal gastric juice at a neutral reaction. The intrinsic factor appears to be neither HCl, nor pepsin, nor trypsin; it is confined to the gastric juice and is destroyed by boiling and behaves like an enzyme [Castle *et al.*, 1931].

In view of what is known of the intrinsic factor, its resemblance to an enzyme, *etc.*, we felt that it would be of value to study more closely the interaction of gastric juice and muscle-protein with the object of obtaining chemical evidence of the production of the active principle. Up to this time the test for the presence of the haematopoietic principle in digests, and incidentally, for the presence or absence of the intrinsic factor in a given sample of gastric juice, has been a reticulocyte response after feeding the products of digestion to cases of pernicious anaemia.

There is evidence that the active principle produced during the digestion of muscle-protein by gastric juice is a water-soluble nitrogenous base, not precipitated by trichloroacetic acid [Cohn *et al.*, 1930]. We therefore planned to determine whether a non-protein nitrogenous body results from such digestion by normal gastric juice *in vitro* near the neutral point, and if so, whether the gastric secretion of cases of pernicious anaemia shows a diminished activity in this respect.

Experimental.

Gastric juice (obtained 1 hour after subcutaneous injection of histamine, or following ingestion of 5 % alcohol) was incubated with muscle-protein in a buffered medium of approximately p_H 6. The unchanged protein was removed by means of trichloroacetic acid and the N in the filtrate determined.

A difficulty which we early encountered was the occasional presence of minute amounts of trypsin in the gastric juice: such tryptic activity was exceedingly small but was sufficient to cast doubt upon our findings. Experiments with commercial trypsin (Liquor Pancreaticus (Armour) precipitated with alcohol, the precipitate washed with alcohol, dried and taken up in saline) showed, however, that the amount of soluble N produced from beef-muscle by this enzyme is 50 % less at p_H 6 than at p_H 8. We were able, therefore, by determining the extent of digestion at p_H 8, to make a suitable correction for tryptic activity when it was present. In doing this we were obviously neglecting the possibility of digestion such as we were seeking occurring at the alkaline reaction, a circumstance which could not be excluded and which, in any case, does not minimise the significance of our findings.

The activity of pepsin under the conditions of our experiments becomes negligible at p_H 3·5, *i.e.* well removed from the region in which we were working.

Method. The gastric juice was freed from solid particles and its reaction adjusted to p_H 6 with alkali or acid as required. 5 ml. were measured into each of three test-tubes: to one of these tubes were added 5 ml. of $N/5$ HCl; to the others 5 ml. of $M/20$ phosphate buffer solution of p_H 6 and 8 respectively. The contents of the tubes were mixed and the reaction checked with the glass electrode. 4 ml. portions of this mixture were separately transferred to test-tubes containing 50 mg. of muscle-protein (this was probably the globulin of muscle and was prepared according to the directions of Castle *et al.* [1931], dried and powdered). The remaining fluid was heated to the boiling-point, cooled, and 4 ml. portions were similarly put up with protein as controls. All the tubes were incubated at 40° for 3 hours and gently agitated 3 or 4 times an hour. When incubation was complete 2 ml. of 25 % trichloroacetic acid were added to each tube, and the tubes after shaking were allowed to stand overnight. Finally, the contents of the tubes were filtered and the N in 4 ml. of the filtrates determined by the micro-Kjeldahl method, using $N/100$ acid. The results represent the differences between the N values of the two digests, calculated as for 100 ml. of gastric juice.

Results.

In Table I our findings in a series of experiments on the gastric juice from normal and pernicious anaemia and other patients are set out. Where the samples of juice contained trypsin, as evidenced by digestion at p_H 8, the amounts of soluble N produced at p_H 6 have been corrected by subtraction of one-half the values obtained at p_H 8.

It will be seen that in all but two cases in the normal group, N amounting to 5–17 mg. per 100 ml. of juice was produced at p_H 6. There is no apparent reason

Table I. *Action of gastric juice on muscle-protein.*

mg. of soluble N per 100 ml. of juice.
(Values at p_H 6 are corrected for trypsin.)

	p_H 1–2	p_H 6	p_H 8
Normals	146	14	9
	227	17	34
	234	13	0
	164	17	11
	200	0	2
	151	7	1
	157	5	2
	174	7	6
	153	11	0
	192	5	0
	156	6	0
	155	11	0
	160	0	0
Pernicious anaemia	78	0	3
	128	0	5
	31	5	6
	30	4	14
	42	13	23
	24	0	5
	*203	1	0
	61	1	3
	24	0	8
Achlorhydric secondary anaemia	99	22	0
	61	4	0
	66	10	5
	—	6	2
Sub-acute combined degeneration of spinal cord	123	8	6

* Free HCl after histamine.

for the failure of two cases to show digestion, and no explanation is possible at this stage.

Turning now to the findings in the collection of cases of pernicious anaemia, with one exception, digestion at p_H 6 was very considerably less than in the normal group: comparison of the two groups clearly shows a difference in the behaviour of the anaemic and normal secretions in regard to the quantities of soluble N produced under the conditions of the experiments.

Of four cases of achlorhydric secondary anaemia, two conformed to the normal and two to the pernicious type.

The amount of N produced at p_H 6 bears no relation to the magnitude of either tryptic or peptic digestion. The juice in pernicious anaemia was weak in peptic activity under these conditions: this is in agreement with the findings of other workers using different substrates.

One case in the pernicious group was found to have free HCl in the gastric juice after histamine and peptic activity above the normal average. The occurrence of free HCl in the gastric secretion in this disease is extremely rare, and it is particularly interesting that in this case there should have been practically no evidence of activity by our method. The blood of the patient, a female, showed R.B.C., 1,400,000; Hb., 36 %; and C.I., 1·3.

In addition we have made some observations of a preliminary nature on the effect of p_H on the production of the soluble N. Samples of normal gastric juice

were allowed to act upon muscle-protein, in a buffered medium, at various reactions. Two curves are reproduced in Fig. 1. In the same figure are plotted curves for the action of pepsin and trypsin under similar conditions.

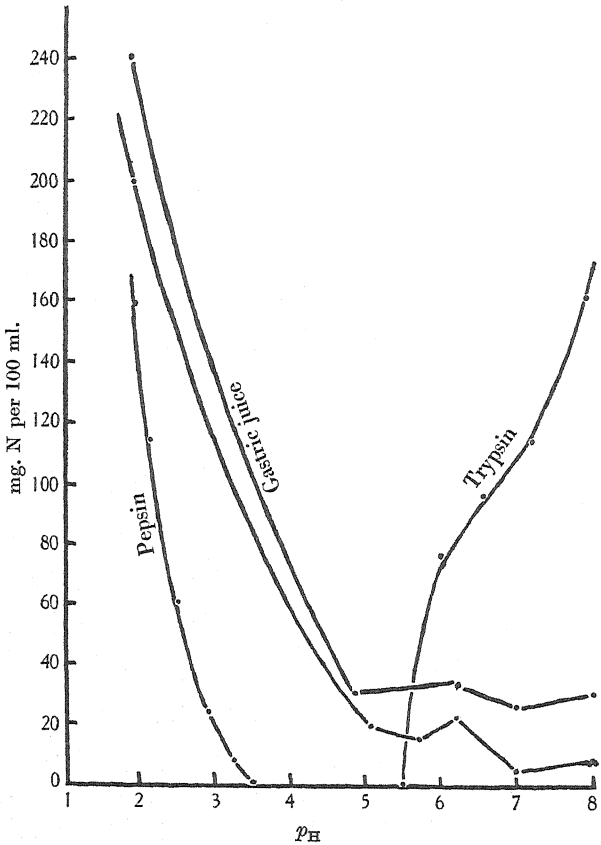

Fig. 1. Action of pepsin, trypsin and gastric juice on muscle-globulin.

Castle *et al.* [1931] found that commercial pepsin was devoid of the intrinsic factor: we assume that the behaviour of the enzyme preparations used by us (pepsin scales, B.P., and Armour's pancreatic extract) is in accordance with that of the gastric enzymes. It will be observed that just as the action of pepsin becomes negligible at p_H 3·5, trypsin ceases to produce any N from muscle-protein at about p_H 5·5, with the result that there exists a range from p_H 3·5 to 5·5 over which neither enzyme yields demonstrable amounts of N under the conditions of our experiments. In regard to gastric juice, however, it is clear that even at reactions within the range p_H 3·5–5·5 definite activity exists, and it would seem to be an advantage in future study to concentrate on this region and so eliminate altogether the participation of pepsin and trypsin.

At this stage we can do little more than record these findings in the hope that subsequent work will reveal their true significance.

We have had in mind the possibility that a bacterial agency might be responsible for the effects obtained. Control experiments in which toluene was

used to inhibit bacterial activity still gave positive results. Further, it is in the anaemias in which one finds a rich bacterial flora in the stomach owing to the absence of free acid that the low results were obtained.

If, as is possible, the nitrogenous substance produced in our experiments during digestion of muscle-protein by gastric juice near the neutral point is related to the haematopoietic principle of Castle, then we have demonstrated in a direct manner the relative absence of the intrinsic factor from the gastric secretion in pernicious anaemia. In consideration of the finding of a certain degree of activity among the anaemias, it must be remembered that even if the gastric juice in pernicious anaemia had all the characteristics of normal juice it is highly probable that its normal functions would be disturbed by a deficiency of secretion, the amount of gastric secretion being so small in this disease. When the secretion is not only scanty in amount but poor in quality it can easily be understood that serious impairment of its function may result.

In regard to the findings in achlorhydric secondary anaemia, it is of much interest, in view of the possibility of a transition from this condition to true pernicious anaemia, that some cases show little activity.

We hope that continuation of this work with improved methods of investigation will lead to a fuller understanding of the significance of Castle's observations and of the rôle of gastric secretion in the workings of the body.

Summary.

1. Small amounts of soluble N are produced when normal gastric juice is incubated with beef muscle-protein at p_H 6.

2. The amounts of this N produced by the gastric secretion from cases of pernicious anaemia were found to be diminished.

3. In the few cases of achlorhydric secondary anaemia studied the results were variable.

4. Pepsin and trypsin play no part in the process.

5. It is possible that the intrinsic factor (Castle) of the gastric juice is concerned in the production of the nitrogenous substance.

It gives me much pleasure to express my thanks to Prof. O. L. V. de Wesselow, Director of the Medical Unit, for suggestions and criticism in the course of this work, and to the Medical Research Council for a part-time grant.

REFERENCES.

Castle (1929). *Amer. J. Med. Sci.* **178**, 748.
—— Heath and Strauss (1931). *Amer. J. Med. Sci.* **182**, 741.
—— and Townsend (1929). *Amer. J. Med. Sci.* **178**, 764.
—— —— and Heath (1930). *Amer. J. Med. Sci.* **180**, 305.
Cohn, McMeekin and Minot (1930). *J. Biol. Chem.* **87**, xlix.
Minot and Murphy (1926). *J. Amer. Med. Assoc.* **87**, 470.

XCIV. STUDIES ON THE PERMEABILITY OF ERYTHROCYTES.

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Kerr [1929] concluded that in solutions outside the physiological range erythrocytes were permeable to Na^+ and K^+. He also observed that ox erythrocytes suspended in an aqueous solution containing 200 mg. K per 100 ml. showed a greater increase in their K content than did cells suspended in serum of which the K content had been raised to 200 mg. per 100 ml. and hence assumed that normal serum lowered the permeability of erythrocytes. In view of the well recognised depressing effect of Ca^{++} on the permeability of a number of animal membranes, *e.g.* in the case of *Arbacia* eggs as described by Lucke and McCutcheon [1928], it was thought to be not improbable that the serum-Ca played an important rôle in determining the impermeability of the erythrocyte membrane to Na^+ and K^+ which is normally observed. The experiments to be described were carried out primarily to test this theory.

Experimental.

Freshly collected ox blood was filtered through muslin, centrifuged, the serum and top layer of corpuscles were removed and 25 ml. of corpuscles suspended in 25 ml. of saline in a corked flask. After a given time the suspension was centrifuged, the saline removed and 5 ml. of corpuscles measured out into a beaker and diluted with distilled water. The proteins were precipitated with 10 ml. of trichloroacetic acid, filtered off and the filtrate was evaporated to dryness in a silica flask. Ashing was completed by Kutz's method [1931]. The residue was taken up with water and made up to 10 ml.

Sodium determination. The volumetric method of Kahane [1930] was tried but found to be quite unsatisfactory. Balint's [1924] modification of the Kramer-Tisdall method gave good results and was employed throughout.

Potassium determination. The method of Kramer [1920] was used.

Blood-volume changes. These were determined by blood counts on the centrifuged corpuscles and by haematocrit determinations on the suspension.

Results.

In Table I are shown the results of suspending ox corpuscles in various aqueous solutions using this procedure. Exp. 1 shows a large decrease in the potassium content of the corpuscles when suspended in 0·9 % NaCl. Addition of $CaCl_2$ up to 100 mg. of Ca per 100 ml., which is roughly ten times as much as occurs in normal serum, produces no significant effect on this decrease. Exp. 2 shows that there is little difference in K content between the control cells and those suspended in NaCl, Ringer solution and buffered Ringer. Now these two experiments differ in the way in which the controls were determined. In Exp. 1, as

Table I.

No.	Washing fluid	Time	K mg./ 100 ml.	Na mg./ 100 ml.	Vol. chge. %	Corr. K	Corr. Na
1	Serum	—	87·9	—	—	87·9	—
	0·9 % NaCl	Overnight	78·9	—	−5	75·0	—
	0·9 % NaCl + 20 mg. Ca/100 ml.	,,	79·1	—	,,	75·1	—
	0·9 % NaCl + 40 mg. Ca/100 ml.	,,	79·5	—	,,	75·5	—
	0·9 % NaCl + 60 mg. Ca/100 ml.	,,	78·9	—	,,	75·0	—
	0·9 % NaCl + 100 mg. Ca/100 ml.	,,	80·0	—	,,	76·0	—
2	Serum*	Overnight	58·5	—	+3	60·2	—
	0·9 % NaCl	,,	63·0	—	−6·3	59·6	—
	Ringer	,,	67·4	—	−13	58·6	—
	Buffered Ringer	,,	68·5	—	−12	60·3	—
3	Serum*	6 hours	61·1	—	+3·2	63·1	—
	NaCl oxalate	,,	61·8	—	−2·9	60·0	—
	Oxalated serum	,,	64·0	—	−7·3	59·3	—
	Ringer oxalate	,,	64·8	—	−9·4	58·7	—
4	Serum	Overnight	86·1	180	—	86·1	180
	Serum*	,,	77·6	196	12·3	68·0	175
	1·5 % KCl	,,	192	155	−6	181	146
	1·5 % KCl + $CaCl_2$	,,	198	154	−9	180	140

* Corpuscles left in contact with their serum.

in Kerr's experiments, the control corpuscles were not left in contact with their serum but were immediately ashed and analysed for potassium, whilst in the second experiment the control corpuscles were left suspended in serum for the same period as the others were left in their respective aqueous solutions. If the corpuscles are impermeable to Na and K when in contact with their serum, then there should be no difference between the values obtained by either method. The fact of the small difference between the control corpuscle-potassium and that of corpuscles suspended in NaCl in the one case and the large difference in the other case would point to the possibility of potassium leaking out of the corpuscles almost as well when in contact with serum as when in contact with an aqueous NaCl solution. Exp. 4 of Table I, in which controls were determined in both ways, confirms this, the K content of corpuscles left overnight in contact with serum in the ice-chest decreasing by 18 mg. per 100 ml. Returning to Exp. 2 it is seen that it is very difficult to draw any definite conclusion as to the effect of suspending corpuscles in Ringer or the effect of buffering the solution, since the differences are small and the error introduced by the volume-change correction would probably cover these differences. Again in Exp. 3 it appears that oxalated serum approximates more closely in behaviour to NaCl solution than to normal serum but the differences are small and come within the maximum error. In 1·5 % KCl an increase of over 100 % in the K content and a decrease of about 30 % in the Na content are observed. The Ca^{++} seems not to affect the permeability of the corpuscle membrane to K^+ or Na^+.

It was now felt necessary to develop a technique in which the error introduced by the blood-volume correction, amounting on the average to about 5 %, could be removed. In the next series of experiments 5 ml. of corpuscles were placed in a centrifuge-tube and 10 or 5 ml. of the saline added. After a given period the corpuscles were centrifuged down, and the supernatant fluid was carefully and completely sucked off, great care being taken not to remove any corpuscles. The whole of the corpuscles in the tube were then laked, ashed and analysed for Na and K. This method is independent of the volume changes, as the whole of the corpuscles are analysed and not an aliquot as previously. Table II shows some of

Table II.

No.	Washing fluid	Time hrs.	K mg./ 100 ml.	Na mg./ 100 ml.
1	Serum	—	71·2	—
	Serum*	4	68·3	—
	0·9 % NaCl	4	65·0	—
	Buffered Ringer	4	67·9	—
2	Serum*	3	84·4	182
	1·5 % KCl	3	146	135
	1·5 % KCl + $CaCl_2$	3	145	136
3	Serum	—	64·7	190
	Glucose	3	63·1	145
4	Serum	—	75·4	174
	84 % serum; 16 % H_2O	2	67·2	136
5	1·5 % KCl	2	151	125
	1·5 % KCl; 0·9 % NaCl	2; 0·5	85·4	163
	1·5 % KCl; 0·9 % NaCl	2; 1	83·7	163
6	Serum	—	—	208
	Glucose	0·5	—	151
	1·5 % KCl	0·5	—	153
	Glucose: KCl, 9 : 1	0·5	—	150

* Corpuscles left in contact with their serum.

the results obtained by this method. Exp. 1 shows that in 0·9 % NaCl alone the corpuscles lose more K than they do in serum, whilst in buffered Ringer the losses are approximately the same. Hence the losses of K in NaCl observed are probably due to a general increase of permeability consequent upon certain changes due to degradation characteristic of dead tissue and not to the removal of any specific factor in the serum.

Losses of Na and K are experienced on suspending the corpuscles in isotonic glucose or dilution of the serum by about 20 %. The large increase in K content and the large decrease in Na content observed on suspending corpuscles in KCl solution might quite reasonably be interpreted as adsorption and desorption phenomena. It may be recalled that the average contents of ox-serum are 20 mg. K and 300 mg. Na per 100 ml. Hence it would be expected that if adsorption occurred at the serum-interface Na would be the chief constituent of this adsorption layer. Thus suspending corpuscles in a medium containing very little or no Na, *e.g.* in glucose or KCl should produce a large drop in their Na content. The succeeding experiments represent attempts to solve this problem.

In Exp. 5 of Table II three lots of corpuscles were suspended for 2 hours in 1·5 % KCl. One lot was then analysed for K whilst the two others were suspended in 0·9 % NaCl. One lot was immediately centrifuged down and the other after half an hour. If the potassium were actually permeating the membrane one might expect to find a greater amount in the lot immediately centrifuged off than in the lot centrifuged half an hour later. The results show no appreciable difference between the two. (It is to be noted that the centrifuging was allowed to continue for half an hour, that time being thought necessary; it was later discovered that from a slightly hypertonic aqueous solution corpuscles could be centrifuged off within 2–3 minutes.) A comparison of the losses of Na in glucose and KCl shows no difference. If the loss were due to desorption one would expect that the desorption would occur more readily in KCl where there are ions to exchange with the adsorbed Na than in glucose. However it must be pointed out that we are not here measuring relative velocities but the amounts

lost in one specified time. In Table III the results of a series of kinetic experiments are shown. Exps. 1 and 2 show the effect of suspending corpuscles for times varying from 8 to 60 minutes in 1·5 % NaCl. As is observed the corpuscle-Na

Table III.

No.	Washing fluid	Time (mins.)	K mg./100 ml.	Na mg./100 ml.
1	Serum	—	83·2	159
	1·48 % NaCl	8	79·7	197
	,,	30	79·6	218
	,,	60	76·0	213
2	Serum	—	77·5	185
	1·53 % NaCl	7	74·2	232
	,,	15	74·2	247
	,,	22	74·0	255
	,,	26·5	73·8	278
	,,	31·5	73·8	276
3	Serum	—	93·7	176
	1·5 % KCl	4·5	261	131
	,,	7·5	265	129
	,,	15·5	271	126
	,,	30·0	288	126
	1·5 % KCl; 0·9 % NaCl	30; 5	148	200
	,,	30; 9	138	174
	,,	30; 10	137	188
	,,	30; 31	136	176
4	Serum	—	—	160
	1·5 % NaCl	8·5	—	226
	,,	11·0	—	228
	,,	2·85	—	231

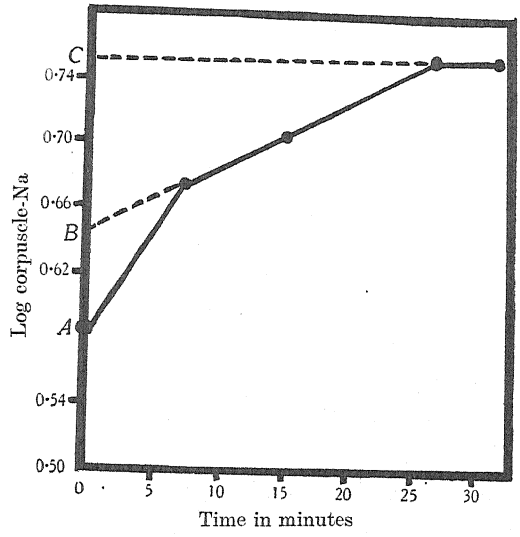

Fig. 1.

increases with time arriving at a stationary value some time between 8 and 30 minutes. The results of Exp. 2 are shown diagrammatically in Fig. 1 where the log of the Na content of the corpuscles is plotted against time. If the process is one of permeation a straight line should result; the graph shows a sharp initial

rise and then a slower one to equilibrium. Exp. 3 shows similar behaviour in 1·5 % KCl and subsequent suspension in 0·9 % NaCl.

Two explanations of Fig. 1 are possible. Either (*a*) the initial sharp increase is due to adsorption, and the slower increase due to penetration, in which case the adsorption would be represented by *AB* and the permeation by *BC*; or (*b*) the effect may be due to inefficient stirring, in which case the whole increase might be due to adsorption or permeation or both. To test this last hypothesis Exp. 4 was performed using twice as much saline for suspending the corpuscles as in Exps. 1, 2 and 3, and the suspension was vigorously stirred for at least 2 minutes before being centrifuged. As the Table shows the phenomenon of the secondary increase has disappeared under these conditions.

In Table IV and Fig. 2 are shown the results of suspending corpuscles in mixtures of NaCl and KCl, the K varying in concentration from 40 to

Table IV.

Washing fluid		Corpuscles	
Na mg./100 ml.	K mg./100 ml.	K mg./100 ml.	Na mg./100 ml.
Serum		84·8	203
39·2	439·5	85·7	207
58·8	427·7	86·1	207
78·4	217·5	89·3	207
117·6	393·0	96·0	207
176·6	358·0	105·0	205
225·0	324·0	113·0	197
298·0	287·0	118·0	189
588·0	116·0	145·0	154

Corpuscles suspended for 1 hour in washing fluid.

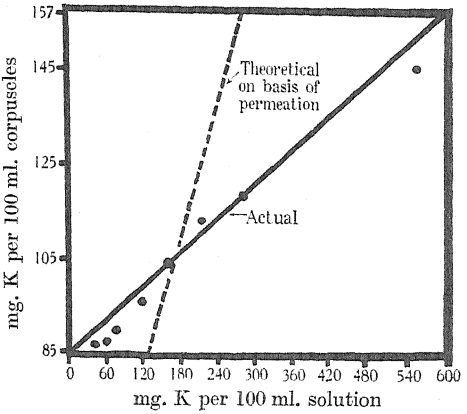

Fig. 2.

600 mg./100 ml. and Na from 440 to 136 mg./100 ml. The total molar concentration of the two ions Na^+ and K^+ was kept constant to maintain identical tonicity of the different solutions and so prevent differences in volume.

Applying the Freundlich adsorption equation:

$$c = kC^{1/n}$$

this may be written:

$$\frac{kC}{C^{\left(\frac{n-1}{n}\right)}} = c,$$

the denominator of the left-hand side representing the factor introduced to account for the limited area of the surface available for adsorption. Applying this to the case where we have both Na^+ and K^+ present:

$$c_{K^+} = \frac{kC_{K^+}}{(C_{K^+} + C_{Na^+})^{\frac{n-1}{1}}},$$

assuming that n is the same for both Na^+ and K^+. Now under the conditions of the experiment $(C_{K^+} + C_{Na^+})$ is constant, so that the adsorption isotherm of either component should be a straight line. It is unfortunate therefore that the shape of the curve of corpuscle-K against concentration in the aqueous solution will not help to distinguish between adsorption and permeation; however, the slope of the curve will tell us whether anything like a simple equilibrium is being established. The actual curve, which has been drawn as a straight line, is certainly rather ambiguous, and one must hesitate before deciding how to characterise the phenomenon it represents. The theoretical curve, assuming that K^+ permeates and is divided equally between the two media, is seen to be very much steeper than the actual curve, showing that if the increases are due to permeation they must occur in accordance with some very obscure equilibrium.

Discussion.

The serum-factor of Kerr appears not to be Ca^{++}. The apparent difference in behaviour between corpuscles in serum and those in NaCl solution has been shown to be illusory, depending on the method of determining the control. K leaks out of the corpuscles into the serum at about the same rate as into NaCl solution. The results of suspending corpuscles in isotonic glucose and diluted serum do not agree with those of Hamburger [1910]. Hamburger found no change in glucose solution and penetration of Na in diluted serum. A point which bears very strongly against the explanation of the losses of Na in KCl and glucose solutions as being due to desorption is the following. If the total molar concentration of Na + K in the corpuscles is compared with that of the serum we find that they are equal; *e.g.* the average of seven different lots of blood gives a total conc. of 14·9 *mM*/100 ml. for the corpuscles and 14·9 *mM*/100 ml. for serum. This of course is to be expected. Now if we assume that approximately 25 % of the corpuscle-Na is adsorbed on the surface, as Exps. 2, 3, 5 and 6 of Table II would indicate, then the average total molar concentration in the corpuscles becomes 13·7 *mM*/100 ml., *i.e.* there would be an osmotic difference between the cells and serum at equilibrium, which of course is impossible. It must therefore be concluded that the losses of Na in KCl and glucose solutions are due to permeation. Hence we are confronted with the phenomenon of a rapid permeation (within 5 minutes) of some 50 mg. Na per 100 ml. to an equilibrium state in which a high concentration gradient is still maintained. From Table IV it is observed that the concentration of Na in the washing fluid may be decreased from 440 to 360 mg. per 100 ml. without producing any significant change in the corpuscle-Na, which seems to indicate that there is a concentration gradient which has to be exceeded before any permeation can occur.

As far as the K^+ ion is concerned a definite but small loss of K occurs in NaCl solution which must be due to permeation. All that can be said of the behaviour of corpuscles in KCl solution is that if permeation of K^+ does occur it takes place within 5 minutes, and an equilibrium position is reached remotely different from that expected on the basis of free permeation.

A possible explanation of these phenomena might be arrived at on the assumption that treatment of the cells with the solution examined itself affects the permeability of the corpuscles, increasing the proportion of those which may be called "dead cells." These "dead cells" will establish a perfect thermodynamic equilibrium with the surrounding fluid, whilst the remainder will maintain their normal impermeability. The net effect will then appear as a partial permeability such as is actually observed. Against this is the fact that only in rare instances was any haemolysis observed, so that a state of permeability to Na^+ and K^+ must be postulated which lies mid-way between those of the normal cell and the truly "dead" cell. Further, one would have to assume that the corpuscles are much more permeable to Na^+ than to K^+ in this hypothetical dead state, since in glucose solutions only about 2 % of the K^+ is lost compared with about 30 % of the Na^+.

Ponder and Saslow [1930; 1931] conclude from their measurements of corpuscular volume that in isotonic salt solutions a loss of osmotic substances occurs, and in hypertonic NaCl and KCl the two salts penetrate to such an extent as to produce an increase in volume after 5 minutes. The results described in this paper show the loss of only small quantities of K in isotonic NaCl and large quantities of Na in KCl accompanied, however, in the latter case by a larger increase in the K content. In 1·5 % NaCl the increases of Na observed are certainly not sufficient to demand an increase in corpuscular volume, and in actual fact an invariable contraction occurred in 1·5 % NaCl and KCl solutions lasting for hours. It must be borne in mind, however, that Ponder and Saslow were working with rabbit's blood, and such large differences in behaviour between species are not out of the question. Reference may here be made to the work of Woodhouse and Pickworth [1932], who measured the permeability of corpuscles to a large number of ions. These authors did not study the influence of K^+ ions as "it may be taken without further experimental proof that the erythrocyte is impermeable to K^+," yet if they had measured the permeability of K^+ they would have obtained a "permeability coefficient" of over 40, a value they obtained for Cl^- in NH_4Cl. Until some method has been devised which will decide unequivocally whether a given ion permeates or is adsorbed one must accept with reserve their statement that the NH^+_4 ion permeates readily.

Summary.

1. In 0·9 % NaCl ox erythrocytes lose K; this loss is unaffected by the presence of Ca^{++} ions and is about equal to the loss sustained in their normal serum under the same conditions.

2. In 1·5 % KCl the corpuscles lose 25–30 % of their Na within 5 minutes; no further appreciable losses occur after this time. The corpuscle-K increases by 100–200 % in the same time and remains constant.

3. In 1·5 % NaCl a considerable increase in corpuscle-Na occurs, but not sufficient to raise the corpuscle concentration to that of the external solution.

4. In glucose solution a large loss of Na and a small loss of K occur. The loss of Na is the same in glucose as in KCl or in mixtures of the two.

5. In diluted serum losses of Na and K are observed.

6. The corpuscle-K is plotted against the concentration of K in the suspending fluid when the latter is varied between 40 and 600 mg./100 ml. The results are not in accord with what would be expected on the basis of ready permeation and only fit approximately an adsorption curve.

My thanks are due to Prof. J. C. Drummond for his kind interest and helpful criticism, to the Department of Scientific and Industrial Research and the Medical Research Council for personal grants during the tenure of which this work was done, and to the trustees of the Dixon Fund for defraying part of the expenses of the research.

REFERENCES.

Balint (1924). *Biochem. Z.* **150**, 424.
Hamburger (1910). *Arch. Int. Phys.* **10**, 1.
Kahane (1930). *J. Pharm. Chim.* (7), **11**, 425.
Kerr (1929). *J. Biol. Chem.* **85**, 47.
Kramer (1920). *J. Biol. Chem.* **41**, 263.
Kutz (1931). *J. Biol. Chem.* **92**, xxii.
Lucke and McCutcheon (1928). *J. Gen. Physiol.* **12**, 127.
Ponder and Saslow (1930). *J. Physiol.* **70**, 169.
—— —— (1931). *J. Physiol.* **73**, 267.
Woodhouse and Pickworth (1932). *Biochem. J.* **26**, 309.

XCV. CALCIFICATION OF HYPERTROPHIC CARTILAGE *IN VITRO*.

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Introduction.

In a previous paper on the experimental calcification of cartilage *in vitro* [Robison *et al.*, 1930] the conclusion was stated that two distinct mechanisms are concerned in normal calcification of cartilage and bone. The first of these, phosphatase-phosphoric ester, brings about the necessary supersaturation of tissue fluid with bone salt [Robison, 1923; 1932]. The second becomes effective only when supersaturation in sufficient degree has been achieved; it provokes the rapid and orderly deposition of the calcium salt in the ground substance of the tissue [Shipley *et al.*, 1926; Robison, 1926].

The share contributed by each mechanism towards the ultimate deposition of the solid calcium salt may depend on the state of the blood with respect to this salt and on the amount of phosphoric ester available.

The distinction between these two mechanisms is empirical. Their activity becomes apparent under different experimental conditions; but it is not affirmed that the mechanisms are entirely independent.

Previous experiments brought to light the favourable effect of bicarbonate on calcification and the inhibitory effect of other ions, notably Mg^{++}, and of protein, and the inhibition of the second mechanism by potassium cyanide, chloroform or by dehydration of the cartilage by acetone *etc.*

Experiments planned to obtain more information on the nature and properties of the dual calcifying mechanism, and particularly of that component which we have termed the second mechanism, are described in this paper.

Experimental.

The technique was in general the same as that previously employed [Robison *et al.*, 1930]. Longitudinal slices cut from the heads of the long bones of rachitic rats were immersed for periods of 16–20 hours in sterile salt solutions at 37° and p_H 7·4. In these experiments the constant-flow apparatus previously described was not used; but the flasks (20 or 100 ml.) containing the bone slices were slowly and continuously rotated at 37° so that all surfaces of the slices were equally exposed to the calcifying solutions. The slices were subsequently fixed, stained with silver nitrate and cleared in cedar wood oil, so that the amount of new calcification in the zone of hypertrophic cartilage could be more readily judged. This is shown in the tables by a figure (maximum 10) representing the extent of the deposit and bars (maximum 5) representing the density, thus $6\equiv$.

[1] Grocers' Company Research Student. Part of the work described in this paper was included in a thesis submitted by A. H. Rosenheim and approved for the degree of Ph.D. in the University of London.

The composition of the basal solution is given in Table I. It differs slightly from that previously used, approximating still more closely to normal plasma in salt content.

Table I. *Composition of experimental solutions.*

g. per litre basal solution		Ion	Millimols per litre: Experimental solution 8 : 5 : 0	Millimols per litre: Normal plasma
NaCl	6·0	Na	130·0	146·0
$NaHCO_3$	2·2	K	5·6	5·1
KCl	0·3	Ca	2·0	2·5
$MgSO_4, 7H_2O$	0·25	Mg	1·0	1·25
		Cl	108·0	101·0
		HCO_3	26·0	26·7
		HPO_4	1·6	1·0
		SO_4	1·0	2·0

The molar concentrations of the acid and basic radicals in a typical inorganic calcifying solution, 8 : 5 : 0[1], and typical values for normal human plasma [Kramer and Tisdall, 1922] are compared in the same table.

Calcium was added to the basal salt solution as $CaCl_2$ and inorganic phosphate as $NaKHPO_4$.

Comparison of the effects of various phosphoric esters on calcification in vitro.

In view of the rôle ascribed to the phosphatase mechanism experiments were undertaken to compare the effects of different phosphoric esters on calcification *in vitro* with that of the syrupy sodium glycerophosphate (chiefly α-ester) which had been generally used. The effect may be dependent on such factors as the rate of diffusion into, or specific adsorption by, the cartilage and on the rate of hydrolysis by the bone-phosphatase.

In these experiments the period of immersion and the concentration of ester were varied, so that any differences in behaviour might be brought out more clearly. The various esters used and the experimental results are shown in Table II. One rat was used for each experiment.

No very marked differences were observed in the effectiveness of the first five esters in the table. The smaller deposits obtained in 5 hours with hexose-monophosphate may possibly signify that this ester diffuses somewhat more slowly than glycerophosphate into the cartilage, although the calcification produced in 18 hours was equally great with both esters.

In the inorganic solutions, in absence of phosphoric ester, no calcification was obtained in periods up to 6 hours. Diphosphoglycerate, which is one of the phosphoric esters of the red blood corpuscles, was ineffective. This ester was not hydrolysed in separate tests with extracted bone-phosphatase; moreover the calcium salt was so insoluble as to be precipitated in the experimental solutions. It is possible, however, that a monophosphoglycerate is also formed in the red corpuscles, and this being readily hydrolysable would prove a suitable substrate for the bone-enzyme. The question is at present being investigated in this laboratory.

[1] The experimental solutions are referred to in this way throughout the text. Thus solution 8 : 5 : 3 contains 8 mg. Ca, 5 mg. P as inorganic phosphate and 3 mg. P as organic phosphate per 100 ml. basal salt solution.

Table II. *The effect of various phosphoric esters on calcification* in vitro.

mg. per 100 ml. Ca	mg. per 100 ml. P inorg.	Phosphoric ester	Conc. of ester mg. P per 100 ml.	Exp. 1 Time (hours) 18	2 5	3 6	4 4	5 6	6 6	7 18	8 18
8	2	0	0	.	.	.	.	.	.	0	.
8	3	0	0	.	.	.	.	.	.	3=	.
8	4	0	0	8≡	0	0	0	0	.	.	6≡
8	4	α-Glycerophosphate	2	.	.	.	0	.	.	.	.
			3	9≡	.	.	.	0	4=	7≡	.
			4	.	.	8=	.	.	.	.	.
			6	9≡	7= 5=	8≡	5=	.	.	.	.
			10	.	.	.	.	5≡	.	.	.
8	4	β-Glycerophosphate	3	9≡	.	.	.	.	.	.	.
			6	9≡	7≡ 7≡	8≡	.	.	.	.	.
8	4	Hexosemonophosphate (mixed ester of fermentation)	3	8≡	.	.	.	.	.	.	.
			6	9≡	1= 2=	.	.	.	.	.	.
8	4	Fructosediphosphate	4	.	.	6–	.	.	.	.	.
			6	.	.	7≡	.	.	.	.	.
8	4	Trehalosemonophosphate	2	.	.	.	0	.	.	.	.
			6	.	.	.	4–	.	.	.	
8	4	Diphosphoglycerate	3	.	.	.	.	0 *p*	.	.	.
			4	.	.	0	.	.	.	.	.
			10	.	.	.	.	0 *p*	.	.	.
8	4	Lecithin	7	.	.	.	.	.	0	.	.
			15	.	.	.	.	.	0	.	.
			30	.	.	.	.	.	0	.	.
8	2	Sphingomyelin	3	.	.	.	.	.	.	0	.
			8	.	.	.	.	.	.	2=	.
8	0	Pyrophosphate	3	.	.	.	.	.	.	.	0 *p*
			4	.	.	.	.	.	.	.	0 *p*
8	2	Pyrophosphate	1	.	.	.	.	.	.	.	0 *p*

p signifies precipitation in the solution during the experimental period.

Lecithin, which is not hydrolysed by the bone-phosphatase, had no favourable effect on calcification; but on the other hand sphingomyelin[1], which is hydrolysed to a slight extent, had some beneficial effect.

Substitution of pyrophosphate for glycerophosphate yielded negative results, since precipitation of the very insoluble calcium pyrophosphate occurred in solutions containing no more than 1 mg. P per 100 ml. in this form.

Experiments with other esters which might function as substrates for the bone-enzyme are in progress.

Deposition of salts other than those of calcium in the hypertrophic cartilage in vitro.

The object of a further series of experiments was to discover whether the second mechanism of hypertrophic cartilage is specific for the bone salt, the complex carbonato-phosphate of calcium, or whether it can also function for other salts of low solubility. The range of possible salts was restricted by the necessity of obtaining solutions sufficiently supersaturated, yet with total ion concentration not unduly removed from that of the tissue fluids.

[1] We are much indebted to Dr O. Rosenheim, who very kindly provided us with a specimen of sphingomyelin.

Attempts to substitute arsenate for phosphate were unsuccessful owing to the high solubility of calcium arsenate. A solution containing 0·025 *M* calcium and as much as 3 *M* arsenate failed to give any precipitate in 24 hours at p_H 7·4.

The replacement of calcium by other alkaline earth metals was more practicable although the phosphates of these metals are more soluble than those of calcium.

In order to find for these experiments the conditions most comparable with those leading to deposition of calcium salt in the cartilage, preliminary tests were undertaken to establish the lowest concentrations of metal and phosphate at which general precipitation occurred in the salt solutions under the experimental conditions. Concentrations slightly lower than this were chosen for the experiments.

For the above tests and subsequent experiments in this group potassium, magnesium and sulphate were omitted from the basal solution and the bicarbonate content was varied as shown in Table III[1].

Table III. *Deposition of Ca, Ba, Sr and Mg salts in the hypertrophic cartilage* in vitro.

17–20 hours; 37°; p_H 7·4.

Metal	Conc.	g. per 100 ml. $NaHCO_3$	mg. per 100 ml. P inorganic	mg. per 100 ml. P organic	Deposition in cartilage: From inorganic solution	Deposition in cartilage: From solution + phosphoric ester
Ca	0·0025 *M*	0·22	3	0	3 =	.
	,,	0·22	2	1	.	4 ≡
Ba	0·01 *M*	0·6	6	0	0	.
	0·005 *M*	0·6	10	0	0	.
	,,	0·6	14	0	0 *p*	.
	,,	1·0	18	0	0	.
	,,	0·05	0	30	.	6 ≡
	,,	0·05	5	10	.	0
	,,	0·05	5	30	.	7 ≡
Sr	0·01 *M*	0·22	10	0	0 *p*	.
	,,	0·6	4	0	0	.
	,,	0·6	8	0	3 ≡	.
	,,	0·6	12	0	8 ≡	.
	0·005 *M*	0·05	12	0	0	.
	,,	0·6	16	0	0	.
	,,	0·6	18	0	3 ≡	.
	0·0025 *M*	0·6	50	0	4 ≡	.
	0·01 *M*	0·05	0	30	.	5 =
	0·005 *M*	0·05	8	4	.	0
	,,	0·05	8	10	.	7 ≡
	0·0025 *M*	0·05	32	2	.	0
	,,	0·05	32	4	.	4 =
Mg	0·1 *M*	0·05	14	0	0	.
	,,	0·05	16	0	0 *p*	.
	,,	0·05	10	4	.	0
	,,	0·05	10	10	.	4 =
	,,	0·05	10	30	.	10 ≡

p signifies precipitation in the solution during the experimental period.

[1] In the case of barium and strontium it was found that as the bicarbonate concentration was raised, progressively higher concentrations of inorganic phosphate could be reached before general precipitation in the solution occurred.

Deposition of barium, strontium and magnesium salts in the hypertrophic cartilage could not at first be realised by agency of the second mechanism alone, that is by immersing the bone slices in inorganic solutions containing these metals in place of calcium. Suitable conditions were at length established in the case of strontium; but deposits of barium and magnesium salts have not yet been obtained in absence of phosphoric ester, even with the highest degree of supersaturation which can be realised short of general precipitation in the solution. On the other hand, deposits of the salts of barium, strontium and magnesium were readily obtained in the hypertrophic cartilage *in vitro* by agency of the bone-phosphatase in presence of phosphoric ester. Typical results are set out in Table III, which shows the relative concentrations required for deposition.

The appearance of the deposits, stained with silver nitrate[1] can be seen in Plate I, Figs. 1–4. Fig. 1 shows a deposit of magnesium salt obtained in solution 0·1 *M* Mg : 10 : 60. The deposit was formed in the matrix of the cells, entirely filling the zone of uncalcified hypertrophic cartilage between the epiphysis and the diaphysis. In appearance it closely resembled that obtained with calcium salts.

Deposits of barium and strontium were usually similar in appearance though sometimes they were less sharply outlined, and occasionally the barium salt was deposited in isolated clusters of relatively large crystals, as seen in Plate I, Figs. 5 and 6.

Attempts were made to obtain deposits of lithium and beryllium salts in the cartilage, but the relatively high solubility of lithium phosphate and the extreme insolubility of beryllium phosphate, carbonate and hydroxide offered considerable difficulties, and no definite evidence of deposition was obtained.

We conclude from these experiments that the second mechanism is not strictly specific for calcium, but that it undoubtedly functions better for the calcium salt than for the others which have been tested. It may be that calcium alone forms a highly complex carbonato-phosphate of solubility so much lower than that of the simple phosphates and carbonates that solutions not supersaturated with the latter may be greatly supersaturated with respect to the complex salt which is ultimately deposited.

Effect of potassium cyanide, of fat solvents and of desiccation on the calcifying mechanisms.

In our previous paper [Robison *et al.*, 1930] it was stated that potassium cyanide strongly inhibited calcification in inorganic solutions but had no appreciable effect in solutions containing phosphoric ester. Further experiments have made it clear that the inhibition of the second mechanism by potassium cyanide is only partial and that calcification may still occur in the more highly concentrated inorganic solutions, particularly if the original activity of the second mechanism in the bone is high. This is illustrated by the results in Table IV. The calcification of the control bone slices in solution 8 : 5 : 0 shows that the second mechanism activity was low in rats *a* and *b* and much higher in rats *c* and *d*. In presence of 0·001 *M* potassium cyanide, calcification was still obtained in this solution in bones from the latter rats but not in those from rats *a* and *b*. The effect of 0·01 *M* cyanide was not markedly greater than that

[1] The deposits of barium, strontium and magnesium salts in the hypertrophic cartilage were also found to stain almost as well as those of calcium with alizarin S. In gelatin models, deposits of the phosphates of calcium, barium, strontium, magnesium, beryllium and lithium stained deep red with this dye, as was shown for calcium and strontium by Cameron [1930].

Table IV. *The effect of potassium cyanide on the calcifying mechanisms.*

18 hours; 37°; p_H 7·4.

mg. per 100 ml.			Control				Potassium cyanide							
							0·0001 *M*		0·001 *M*				0·01 *M*	
Ca	P inorg.	P org.	*a*	*b*	*c*	*d*	*c*	*d*	*a*	*b*	*c*	*d*	*c*	*d*
8	5	0	1≡	1=	5≡	9≡	2=	6≡	0	0	1–	4≡	2=	2=
8	6	0	.	.	5≡	.	6≡	.	.	.	2=	.	3=	.
8	3	10	.	.	7≡	.	6≡	.	.	.	7≡	.	.	.

of 0·001 *M*. No inhibitory effect was observed in solution 8 : 3 : 10, containing phosphoric ester.

The previous conclusion regarding inactivation of the second mechanism by treatment of the bone slices with acetone, alcohol or chloroform, or by desiccation, probably requires similar modification. It would seem that the second mechanism, though greatly weakened, is not entirely destroyed by such treatment.

Effect of various organic substances on the calcifying mechanisms.

Calcification both in presence and absence of ester was strongly inhibited by 0·5 % phenol and by 0·3 % thymol; but 0·1 % phenol had little apparent effect. Neither ethylurethane in 1 % solution nor guanidine in 0·1 % solution appeared to influence the calcifying mechanisms.

Inactivation of the calcifying mechanisms by heat[1].

Experiments were undertaken to determine the effect on the two calcifying mechanisms of heating the bone slices at different temperatures. The slices were placed in bicarbonate-free solution 8 : 2 : 0 and maintained at temperatures between 45° and 60° for periods of 10, 15 and 20 minutes. The effect on the two mechanisms was judged by the subsequent calcification of the slices in solutions 8 : 5 : 0 and 8 : 3 : 10, the deposits being compared with those in unheated control slices. No effect on either mechanism was observed after 20 minutes at 45°; but some reduction in the activity of the second mechanism and probably also in that of the phosphatase was apparent after 15 minutes at 48°. The loss of activity was greater at 50° and 55°; but the results were too irregular to justify any conclusion as to the relative rates of destruction. Both mechanisms, however, appeared to be inactivated in 10 minutes at 60°.

Inactivation of the calcifying mechanisms by acid and alkali.

Unsuccessful attempts were made to inactivate the two mechanisms differentially by soaking bone slices for one hour in isotonic salt solutions at different p_H levels. Little effect on either mechanism was noted between p_H 5·4 and 8·9 at 37°; but both were inactivated, at least partially, at p_H 5.

In 1 % ammonia both mechanisms were destroyed in one hour at room temperature. It may be added that preparations of bone-phosphatase are very rapidly inactivated at p_H values below 5·2 or above 10. It is not known to what extent the tissue may be able to maintain an internal p_H differing from that in the surrounding medium.

[1] Other experiments bearing on this question and those discussed in the following two sections have been carried out by Niven and Robison using embryonic rabbit femora. The results are in course of publication.

Survival of the calcifying mechanisms in excised bone slices under various conditions.

The question of the survival of the calcifying mechanisms in cartilage after excision from the body and immersion in various solutions was investigated on account of its theoretical interest and its bearing on the interpretation of experimental results. Some typical experiments are summarised in Table V. The control (0 hour) bone slices from each rat were placed directly in the calcifying solutions 8 : 5 : 0 and 8 : 3 : 10 at 37° and left there for 18 hours. The other slices were first immersed for varying periods in salt solutions, or distilled water, at 0°, 20° or 37° as specified in Table V. They were then transferred to the calcifying solutions for an 18-hour period at 37°.

Table V. *Gradual inactivation of the calcifying mechanisms in bone slices immersed in various solutions at different temperatures.*

Preliminary immersion				Subsequent calcifying solution	Duration of preliminary immersion (hours)						
Rat	Solution	$NaHCO_3$ %	Temp. ° C.		0	½	1	2	4	24	48
					Subsequent calcification in 18 hours; 37°; p_H 7·4						
a	A	0·22	0	8 : 5 : 0	7≡	.	.	.	.	2≡	0
				8 : 3 : 10	7≡	.	.	.	.	3≡	0
b	B_1	0·03	,,	8 : 5 : 0	7≡	.	.	.	.	2=	0
				8 : 3 : 10	7≡	.	.	.	.	3≡	0
c	A	0·22	20	8 : 5 : 0	8≡	7≡	.	6≡	5≡	.	.
				8 : 3 : 10	8≡	.	.	.	9≡	.	.
	,,	,,	37	8 : 5 : 0	8≡	6≡	.	6≡	4≡	.	.
				8 : 3 : 10	8≡	.	.	.	6≡	.	.
d	,,	,,	,,	8 : 5 : 0	5≡	.	.	.	3≡	0	.
				8 : 3 : 10	5≡	.	.	.	5≡	3≡	.
e	B	0·22	37	8 : 5 : 0	6≡	.	6≡	5≡	.	.	.
	B_1	0·03	,,	8 : 5 : 0	6≡	.	6≡	3≡	.	.	.
	B_2	0	,,	8 : 5 : 0	6≡	.	6≡	0	.	.	.
	B	0·22	37	8 : 5 : 0	2=	.	2=	.	.	.	.
				8 : 3 : 10	8≡	.	7≡	.	.	.	.
	B_2	0	,,	8 : 5 : 0	2=	.	0	.	.	.	.
				8 : 3 : 10	8≡	.	7≡	.	.	.	.
g	B_1	0·03	37	8 : 5 : 0	.	5≡	5≡	2=	.	.	.
				8 : 3 : 10	.	.	8≡	.	.	.	.
Distilled water		0	,,	8 : 5 : 0	.	2≡	0	0	.	.	.
				8 : 3 : 10	.	.	5≡	.	.	.	.

Solutions used for the preliminary immersion:

A, 8 : 2 : 0, similar in composition to the calcifying solution but of lower P content. (This solution can itself effect calcification in bones with very exceptionally high second mechanism activity.)

B, basal solution (Table I) containing neither Ca nor P.

B_1 and B_2, similar to B in all respects except bicarbonate content.

In the last experiment (rat *g*) the bone slices in solution B_1 served as controls for those in distilled water.

The results show that when the bone slices were kept at 37° in solution 8:2:0 or in the basal salt solution there was a gradual decrease in the activity of the second mechanism, which became pronounced after 24 hours' immersion. This loss of activity occurred also, though more slowly, at lower temperatures. In salt solutions containing no bicarbonate and in distilled water the inactivation of the second mechanism was more rapid.

These adverse conditions would appear to have had little effect on the phosphatase mechanism over short periods; but calcification in solution 8:3:10 was reduced after prolonged immersion.

These results indicate that in experiments on calcification *in vitro* undue prolongation of the period in the calcifying solutions at 37° can be of little advantage owing to the gradual decrease in the activity of the calcifying mechanisms. Practical experience has, indeed, shown that the maximum calcification obtainable in any given solution at 37° occurs, as a rule, within 18 hours. At room temperature, on the other hand, in solutions 8 : 10 : 0 and 8 : 4 : 10 calcification has been obtained in 43 hours though absent after 19 hours.

Effect of various vitamin and hormone preparations on calcification in vitro.

Experiments were performed to discover whether vitamin D or hormones, which are known to influence the growth and calcification of the skeleton *in vivo*, would have any demonstrable action on the calcification or decalcification of bone slices *in vitro*. The results of these experiments were all negative and will therefore only be summarised briefly.

Vitamin D. (1) Bone slices from a rachitic rat were moistened with 8 : 2 : 0 solution and irradiated in quartz tubes for periods of 15, 30 and 60 minutes before immersion in 8 : 3 : 0, 8 : 5 : 0 and 8 : 3 :10 solutions for 18 hours.

(2) Bone slices from a rachitic rat were immersed for one hour in an olive oil solution of irradiated ergosterol (radiostol) before immersion in the calcifying solutions.

(3) Bone slices were immersed in 20 ml. of various calcifying solutions (with and without ester) in which 0·1, 0·2 or 0·3 ml. of radiostol was emulsified by shaking. A control solution containing 0·2 ml. of olive oil in emulsion was used in addition to the usual controls.

(4) In other experiments calciferol was used instead of radiostol. It was added to the calcifying solutions in amounts equivalent to 400–40,000 antirachitic units per 100 ml. In all the above experiments, untreated slices from the same rat were immersed in similar calcifying solutions (containing no vitamin preparation) to serve as controls. There was no indication of any effect, beneficial or otherwise, of irradiation or of radiostol or calciferol on calcification *in vitro* either in presence or absence of phosphoric ester.

Parathyroid hormone. (1) Bone slices were immersed for 19 hours at 37° in calcifying solutions with and without ester, and containing in addition 10, 20 and 40 units of Eli Lilly parathormone per 100 ml. No increase or inhibition of calcification was apparent; and it was almost impossible to determine by inspection of the stained slices whether there had been any resorption of the previously calcified shaft trabeculae during the experimental period.

(2) Whole bones, heads of bones or half bones of normal young rats were each immersed in 5 ml. of sterile basal salt solution without calcium or phosphate, half the solutions containing 60 units of parathormone per 100 ml. The vessels containing the bones were rotated for 19 hours at 37°. The very slight increase in the inorganic and organic phosphate and the calcium content of the solutions, which occurred during the experiments, was no greater when parathyroid extract was present than when it was not. There was thus no evidence that calcium or phosphate was brought into solution in any form.

Pituitary extract. In two experiments no definite effect on calcification was observed as a result of adding standard posterior lobe extract (equivalent to

0·1 to 15 I.U.) to calcifying solutions with and without phosphoric ester. Short periods of immersion (3 to 6 hours) were chosen in order that any increase in permeability due to the action of the hormone might be more apparent.

Testicular extract. Experiments similar to the above were carried out with testicular extract, for which we are indebted to Drs D. McClean and W. T. J. Morgan. No indication was obtained of more rapid calcification as a result of increased permeability.

Effect on calcification in vitro *of other substances present in the blood.*

The results of two experiments quoted in Table VI show that neither glycine nor urea in concentrations covering the normal physiological range had any significant effect on the activity of the second mechanism. The amounts of new deposit in tibia slices immersed in solutions 8 : 4 : 0 and 8 : 5 : 0 were nearly the same in presence of these substances as in the controls.

Table VI. *The effect of urea and glycine on calcification* in vitro.

17 hours; 37°; p_H 7·4.

	mg. per 100 ml.				mg. per 100 ml.					
					Urea			Glycine		
Rat	Ca	P inorg.	P org.	Control	30	50	60	20	40	50
a	8	4	0	5 =	2 ≡	5 ≡	.	7 ≡	2 =	.
	8	5	0	6 ≡	7 ≡	4 ≡	.	7 ≡	7 ≡	.
b	8	4	0	1 =	.	.	< 1 −	.	.	< 1 −
	8	5	0	5 ≡	.	.	6 ≡	.	.	5 ≡
	8	5	0	4 ≡	.	.	5 ≡	.	.	4 ≡

Table VII. *Effect of sugars and sugar alcohols on calcification* in vitro.

	mg. per 100 ml.				mg. per 100 ml.					
					Glucose		Fructose	Sucrose	Dulcitol	Erythritol
Rat	Ca	P inorg.	P org.	Control	50	100	100	100	100	100
a	10	3	0	5 =		0	.	.	.	.
	10	5	0	7 ≡		0	.	.	.	.
b	10	3	0	1 −		.	.	.	.	.
	10	4	0	4 =	0	< 1 −	.	.	.	.
	10	5	0	.	4 ≡	0	.	.	.	.
	10	6	0	.	4 ≡	2 =	.	.	.	.
	10	7	0	.	6 ≡	5 ≡	.	.	.	.
c	8	4	0	2 =	.	0	3 −	4 =	.	.
	8	5	0	4 ≡	.	0	2 ≡	2 ≡	.	.
d	8	4	0	4 ≡	.	0	.	.	3 ≡	3 =
	8	5	0	5 ≡	.	< 1 −	.	.	5 ≡	5 ≡

On the other hand the results set out in Table VII show that glucose in concentrations of 50 or 100 mg. per 100 ml. had a very definite inhibitory effect on calcification in inorganic solutions. Thus in rat *b* calcification was less extensive in 10 : 6 : 0 solution containing 100 mg. glucose per 100 ml. than in 10 : 4 : 0 solution containing none. In another paper [Rosenheim, 1934, 1] it is shown that this inhibition is exerted also in presence of protein and is additive to the effect of the latter.

It was at first thought that this inhibition might be explained by the combination of the glucose with the calcium ions; but as will be seen from Table VII

no such marked effect could be demonstrated with other sugars or sugar alcohols. Glycogen also, in concentration of 150 mg. per 100 ml., did not affect the amount of calcification in solution 8 : 5 : 0.

Effect of fluoride, iodoacetate and arsenate on calcification in vitro.

The results given in Tables VIII and IX show that both sodium fluoride and sodium iodoacetate exert a most pronounced inhibitory effect on the second mechanism. The former in concentration as low as 0·0001 *M* completely prevented calcification even in solutions 8 : 6 : 0 and 8 : 6 : 10, though calcification still occurred in solution 8 : 6 : 30. In still lower concentration (0·00001 *M*)

Table VIII. *Effect of sodium fluoride on the calcifying mechanisms.*

18 hours; 37°; p_H 7·4.

mg. per 100 ml.			Control			Concentration of NaF: 0·00001 *M*			Concentration of NaF: 0·0001 *M*		
Ca	P inorg.	P org.	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
8	5	0	5≡	4≡	4=	0	0	0			
8	6	0	.	.	.	4≡	0	0			
8	3	10	8≡	7≡	5≡	.	0	.			
8	5	10	.	.	.	7≡	.	0			
8	6	10	.	.	.	.	0	.			
8	6	30	.	.	.	.	.	8≡			

The results given in columns *a*–*c* were obtained with bone slices from three different rats.

Table IX. *Effect of sodium iodoacetate on the calcifying mechanisms.*

18 hours; 37°; p_H 7·4.

mg. per 100 ml.			Control						Concentration of iodoacetate: 0·0001 *M*	0·001 *M*				
Ca	P inorg.	P org.	*a*	*b*	*c*	*d*	*e*	*f*	*b*	*a*	*b*	*c*	*d*	*e*	*f*
8	5	0	6≡	5≡	4≡	5≡	6≡	5≡	<1–	0	0	0	.	3=	3≡
8	6	0	.	.	.	.	.	.	.	.	.	0	0	.	3≡
8	3	10	6≡	8≡	.	6≡	6≡	.	6≡	0	<1–	.	.	6≡	4=
8	5	10	.	.	9≡	.	.	.	.	.	.	0	.	.	6=
8	6	10	.	.	.	.	.	.	.	.	.	0	2=	.	.
8	6	30	.	.	9≡	.	.	.	.	.	.	5–	7≡	.	.

The results given in columns *a*–*f* were obtained with bone slices from six different rats.

sodium fluoride prevented calcification in 8 : 5 : 0 solution although not in solutions 8 : 6 : 0 and 8 : 5 : 10. The inhibitory effect of sodium iodoacetate, although not apparent in such great dilution, was also very marked. In a concentration of 0·001 *M* it prevented or greatly reduced the calcification in the inorganic solutions, while in presence of ester the amount of deposit was generally reduced.

In separate experiments, summarised in Table X, it was shown that neither fluoride nor iodoacetate in concentrations higher than those used in the above experiments affected the rate of hydrolysis of β-glycerophosphate by a preparation of purified bone-phosphatase.

The presence of 0·001 *M* sodium arsenate in the calcifying solutions 8 : 5 : 0 and 8 : 3 : 10 had neither favourable nor inhibitory effect on calcification *in vitro*.

Table X. *Hydrolysis of β-glycerophosphate by bone-phosphatase in presence of fluoride and iodoacetate.*

		Sodium fluoride		Sodium iodoacetate	
	Control	0·001 *M*	0·01 *M*	0·001 *M*	0·01 *M*
Inorganic phosphate (mg. P) liberated in 1 hour at 37° and p_H 8·6 by 1 mg. phosphatase	0·285	0·275	0·28	0·28	0·275

Each value is the mean of four determinations.

Discussion.

Although the work described in this and the succeeding papers has not yielded the exact knowledge of the second mechanism which we sought, the results have enabled us to extend and to correct our previous information as to the qualities of this mechanism and its relationship to phosphatase, while new ideas have emerged which may usefully form a basis for further experiment. A brief survey may be attempted.

The phosphatase hypothesis in its simplest form assumed that the blood is normally in equilibrium with the bone salt and that a condition of supersaturation, which is the theoretical requirement for precipitation of this salt, is produced locally in hypertrophic cartilage and osteoid by the phosphatase of these tissues acting on phosphoric esters derived from the blood. That calcification could be effected in this way was made clear by experiments *in vitro*; while further evidence of various nature emphasised the intimate connection of phosphatase with bone development.

But it was apparent that this simple scheme is not in itself sufficient. A difficulty arises from the presence of phosphatase in many tissues other than bone. It is true that phosphatases are required for other physiological processes as well as for calcification; it is true also that the phosphatase activity of the bone is high and that it is highest at the period when calcification is most active; but the question remains, if the enzyme in these other tissues sets free inorganic phosphate in a fluid already saturated with the calcium salt, why is this salt not deposited in these tissues as it is in bone? Certain of these tissues are liable to become calcified in abnormal or pathological conditions and the relation of this tendency to the distribution of phosphatase is being investigated [Rosenheim and Robison, 1934; Macfarlane *et al.*, 1934]. We incline to believe that the effect of the enzyme in such cases can, at most, be supplementary to other factors.

Another difficulty is that solutions may be considerably supersaturated with the bone salt and yet remain relatively stable. Unpublished experiments have shown that materials of different types may be immersed in such solutions for several days without any deposit being formed upon them. Clearly, some further qualities, differentiating hypertrophic cartilage and osteoid from other tissues, were required; and the experiments of Shipley *et al.* [1926], fully confirmed in this laboratory, proved that such qualities exist.

In our previous paper [Robison *et al.*, 1930] we showed how the calcifying power attributable to these special qualities of hypertrophic cartilage and that due to the phosphatase mechanism could be separately demonstrated *in vitro* under suitable experimental conditions. An empirical distinction between the two mechanisms was thus established but this did not exclude the possibility of their intimate relationship. For the present it is convenient to discuss them

as separate components of a dual system by which the normal process of calcification is effected in cartilage and bone.

We have evidence that both mechanisms arise in hypertrophic cartilage and osteoid tissue and reach their highest activity with the full histological development of these tissues, thereafter becoming weaker as tissue degeneration proceeds[1] [Fell and Robison, 1929; 1930; Rosenheim 1934, 2]. It has been shown also that the power of the second mechanism varies considerably in the cartilage of different animals of the same species and even of the same litter and that this variation is connected in some way with the condition of the animal; it may possibly be related to its store of vitamin D, although at present the evidence for this is slight and indirect [Rosenheim, 1934, 2].

The second mechanism appears to be more sensitive than the phosphatase to various agents and conditions. It is greatly weakened by potassium cyanide, by treatment of the cartilage with various solvents or by its desiccation. It is not, however, entirely destroyed by these agencies, for even after such treatment the cartilage retains a greater tendency to become calcified in highly supersaturated inorganic solutions than is ever possessed by the aorta or kidney. The activity of the second mechanism also declines more rapidly than that of the phosphatase after the bones are removed from the body and the slices placed in the experimental salt solutions at 37°. In distilled water or in absence of bicarbonate the decline is still more rapid but is retarded in the salt solutions at lower temperatures. These facts suggest that the second mechanism is bound up with some labile structure of the tissue colloids but does not depend for its functioning on the living cell.

Many of the experimental findings could be readily explained by attributing to this mechanism the properties of a trigger, its effect on the supersaturated tissue fluid being similar to that of a crystal of sodium sulphate on the supersaturated solution of this salt. Conceivably, surface forces at fibrillar interfaces might exert some such action, though experiments in this direction have given only negative results.

Another suggestion [Robison and Soames, 1924] that the p_H within the cartilage is raised above that of the surrounding medium, also lacks experimental support.

Some of the results recorded in this paper suggest strongly that the second mechanism is, like the phosphatase, enzymic in nature; while none of the experimental data is inconsistent with this view. In particular the very pronounced inhibitory action of sodium iodoacetate and sodium fluoride recalls the effect of these substances on the enzymes of yeast and muscle, preventing the fermentative breakdown of phosphoric esters in the cycle of changes.

Prior to these experiments with iodoacetate and fluoride Harris [1932] had drawn attention to the parallelism between the presence of phosphatase and the storage of glycogen in cartilage and had put forward the very interesting suggestion that the chemical system in the ossification process might bear some relation to that in yeast and muscle cells. He suggested that the "hypertrophic cartilage cells provide both the phosphatase enzyme and the glycogen; the latter on hydrolysis yields hexosephosphoric esters which, under the action of the phosphatase and the calcium of the circulating body fluids, lead to the deposition of an insoluble phosphate of calcium in the matrix." Fell and Robison [1933] pointed out that phosphatase is also actively secreted by the osteoblasts which, according to Harris, are devoid of glycogen; but they considered that these facts are not necessarily inconsistent with his suggestion.

[1] Other experiments bearing on this point will shortly be published by Fell and Robison.

It may be that the bone is not entirely dependent on the blood for its supply of phosphoric esters, but that these are also synthesised in the hypertrophic cartilage and osteoid as a stage in the building-up and breaking-down of glycogen. A cycle of synthesis and subsequent hydrolysis in the matrix could obviously serve as a mechanism for raising the level of inorganic phosphate at the site of calcification. Phosphatase might thus prove to be an essential component of the second mechanism, the whole consisting of a complex system analogous to that in yeast and muscle. Part of this system might be inactivated by such agencies as acetone or desiccation, or inhibited by potassium cyanide, iodoacetate or fluoride; while the phosphatase, being unaffected, could still function if phosphoric esters were supplied. To these suppositions a parallel might readily be found in the effect of various agents on the fermentative complex of yeast.

It may be noted that while calcification *in vitro* of hypertrophic cartilage can be effected by agency of the phosphatase mechanism when the second mechanism has been inhibited or destroyed, the converse, calcification by second mechanism activity after destruction of phosphatase, has never been demonstrated. Further, it has been shown that tissues such as kidney may possess a high phosphatase activity without the second mechanism, but no tissue has been found which possesses an active second mechanism without phosphatase.

On the whole we may say that the results of our experiments emphasise the possible enzymic nature of the second mechanism and its close relationship with the phosphatase. They justify certain comparisons with the enzyme systems of yeast and muscle and thus lend support to the stimulating suggestion of Harris. The value of this suggestion may not necessarily depend on the proof that glycogen storage precedes calcification—a contention concerning which there appears to be some doubt.

The analogy between the systems of bone and muscle cannot be pressed too far, since muscle does not contain the bone-phosphatase nor does it become calcified. Nor does the more general conception of the second mechanism as an enzyme system in any way preclude the possibility that factors of a different type, such as surface forces in the colloidal matrix, may also assist in promoting the deposition of calcium salts from the tissue fluids after supersaturation has been achieved by enzyme action.

The experiments on the deposition *in vitro* of barium, strontium and magnesium salts show that the second mechanism is not entirely specific for the calcium salt of bone; but they also indicate that the properties of this calcium salt are very specially appropriate to the function which it fulfils. Although magnesium is a normal constituent of bone and teeth, the very high concentration of this element found necessary for its deposition *in vitro* provides evidence that in the body it is precipitated as part of a much less soluble complex salt in which it replaces an equivalent amount of calcium.

The inhibitory influence of proteins and of magnesium and other ions on calcification may probably be explained by their effects on the ionisation of calcium and phosphate, though the possibility of some direct action on the second mechanism is not excluded. It is not so easy to account for the marked inhibitory action of glucose which has also been investigated by zu Hörste [1932]. The reason may perhaps be bound up with the possible relation of the second mechanism to carbohydrate breakdown.

The beneficial effect of bicarbonate on calcification in inorganic solutions was considered in our previous paper to supply some evidence that the deposited bone salt is a complex carbonato-phosphate. The present experiments suggest

an additional or alternative explanation—that the presence of bicarbonate in the calcifying solutions favours survival of second mechanism activity. Bicarbonate may serve to neutralise lactic or other acids produced by the tissue enzymes, thus preventing a rapid fall in the internal p_H of the cartilage. Before accepting this explanation we must know to what extent the internal p_H is independent of that of the external medium, since the bicarbonate-free solutions which failed to produce calcification did not themselves show any significant fall in p_H, and since immersion of the bone slices in solutions of p_H 5·4 for one hour was not found to affect the calcifying power of the cartilage. The question of the internal p_H of the cartilage and osteoid matrix bears so directly on the problems of calcification that some method by which it could be measured immediately before calcification begins and without injury to the calcifying mechanism would be of the greatest value.

The gradual decline in the calcifying power of the bone slices after excision, and particularly in the activity of the second mechanism, points to a limitation of this experimental method of studying calcification *in vitro*. To counterbalance the effect of this loss of power, somewhat higher concentrations of the essential ions than are adequate for progressive calcification in the body may be required for deposition in the restricted period *in vitro*, so that direct comparison of the limiting concentrations *in vitro* with those *in vivo* may be misleading. The method remains, however, most valuable for the comparative study of factors which may influence either the concentrations of these ions or the activity of the mechanisms.

Summary.

1. The nature and properties of the dual calcifying mechanism of hypertrophic cartilage have been further investigated.

2. It was shown that α- and β-glycerophosphates, hexosemonophosphate, fructosediphosphate and trehalosemonophosphate are satisfactory substrates for the bone-phosphatase in calcification *in vitro*. Diphosphoglycerate and lecithin were not effective.

3. Deposits of the salts of barium, strontium and magnesium were obtained in the hypertrophic cartilage *in vitro* by the phosphatase mechanism. Deposits of strontium salts were also obtained from highly supersaturated inorganic solutions containing no phosphoric ester. The deposits were formed in the matrix of the hypertrophic cartilage and usually resembled those of calcium salts. The second mechanism is, therefore, not entirely specific for the calcium salt of bone.

4. Calcification occurred in presence of 0·001 *M* potassium cyanide in bone slices immersed in highly concentrated inorganic solutions when the original second mechanism activity of the bone was high. The inhibition of the second mechanism by potassium cyanide previously reported is, therefore, not complete.

5. The two components of the calcifying mechanism could not be inactivated differentially by heating the bone slices at different temperatures or by soaking them in isotonic salt solutions at different p_H levels.

6. A gradual decline in the activity of both mechanisms occurred when the excised bone slices were immersed in various salt solutions at 37°. This decline was more rapid for the second mechanism than for the phosphatase. It was more rapid also in absence of bicarbonate or in distilled water, but was retarded at lower temperatures.

7. Vitamin D or hormone preparations known to influence the growth and calcification of the skeleton *in vivo* had no demonstrable action on the calcification or decalcification of bone slices *in vitro*.

8. Glucose in concentrations of 50–100 mg. per 100 ml. had a very definite inhibitory effect on calcification *in vitro* in inorganic solutions, but no such effect could be demonstrated for fructose, sucrose, dulcitol or erythritol. Glycine and urea, also, in concentrations covering the normal physiological range were without significant effect on the activity of the second mechanism.

9. Sodium fluoride in concentrations as low as 0·00001 *M* and sodium iodoacetate in concentration of 0·0001 *M* exerted a pronounced inhibitory effect on the second mechanism, but not on the phosphatase.

10. The nature of the dual calcifying mechanism is discussed in the light of these and other experimental results.

REFERENCES.

Cameron (1930). *J. Path. Bact.* **33**, 929.
Fell and Robison (1929). *Biochem. J.* **23**, 767.
—— —— (1930). *Biochem. J.* **24**, 1905.
—— —— (1933). *Nature*, **131**, 62.
Harris (1932). *Nature*, **130**, 996.
zu Hörste (1932). *Monatsch. Kinderheilk.* **52**, 439.
Kramer and Tisdall (1922). *J. Biol. Chem.* **53**, 241.
Macfarlane, Patterson and Robison (1934). *Biochem. J.* **28**, 720.
Robison (1923). *Biochem. J.* **17**, 286.
—— (1926). *Biochem. J.* **20**, 388.
—— (1932). Herter Lectures (New York).
—— Macleod and Rosenheim (1930). *Biochem. J.* **24**, 1927.
—— and Soames (1924). *Biochem. J.* **18**, 740.
Rosenheim (1934, 1). *Biochem. J.* **28**, 699.
—— (1934, 2). *Biochem. J.* **28**, 708.
—— and Robison (1934). *Biochem. J.* **28**, 712.
Shipley, Kramer and Howland (1926). *Biochem. J.* **20**, 379.

DESCRIPTION OF FIGURES IN PLATE I.

Figs. 1 to 4. Deposits of magnesium and strontium salts in the hypertrophic cartilage zone stained with silver nitrate.

Figs. 5 and 6. Crystalline deposit of barium salt unstained.
X = deposit of Mg, Sr or Ba salt formed *in vitro*.

Fig. 1. Microtome section of a tibia slice after immersion for 17 hours in solution 0·1 *M* Mg : 10 : 60 containing high concentrations of magnesium and phosphoric ester. Note the extensive deposit of magnesium salt in the matrix of the previously uncalcified hypertrophic cartilage zone. Magnification ×20.

Fig. 2. Tibia slice after immersion for 19 hours in the inorganic solution 0·01 *M* Sr : 12 : 0. Note the dense deposit of strontium salt formed in the hypertrophic cartilage zone in absence of phosphoric ester. Magnification ×8.

Fig. 3. Microtome section of part of a tibia slice after 19 hours in the solution 0·005 *M* Sr : 8 : 30, containing phosphoric ester. Magnification ×25.

Fig. 4. Deposit of strontium salt formed in 19 hours in solution 0·005 *M* Sr : 0 : 30. The appearance of the deposit in the matrix of the hypertrophic cartilage cells closely resembles that of a normal deposit of calcium salt. Magnification ×160.

Fig. 5. Barium salt deposited in crystalline form in the hypertrophic cartilage zone of the tibia of a rachitic rat immersed for 19 hours in solution 0·005 *M* Ba : 5 : 30. Magnification ×25.

Fig. 6. The same crystals photographed by polarised light between crossed Nicols. Magnification ×50.

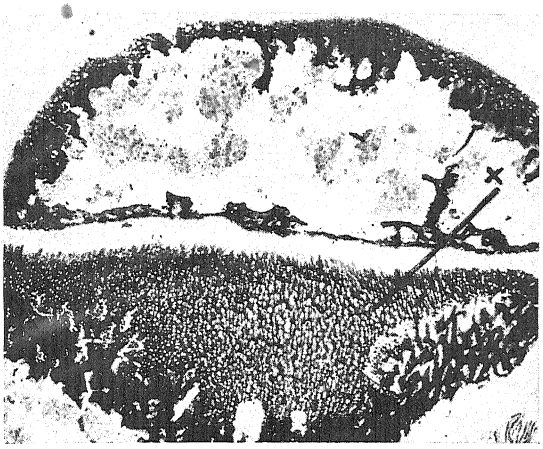

Fig. 1.

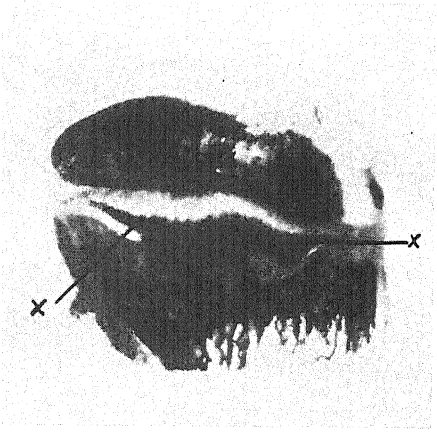

Fig. 2.

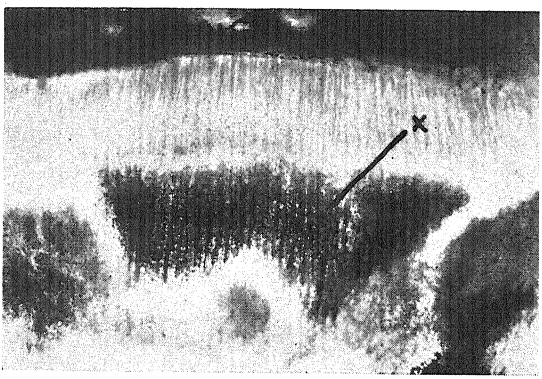

Fig. 3.

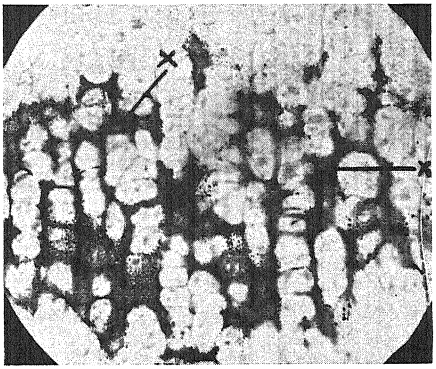

Fig. 4.

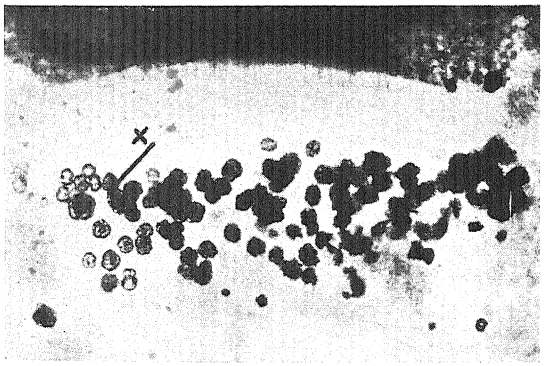

Fig. 5.

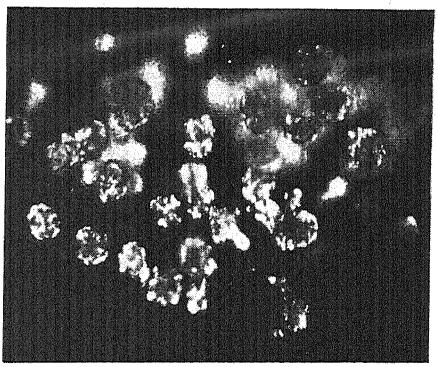

Fig. 6.

XCVI. THE EFFECT OF SERUM-PROTEINS ON CALCIFICATION *IN VITRO*.

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The inhibitory effect of egg-albumin on the calcification of bone slices in organic salt solutions was noted by Shipley *et al.* [1926]. The concentrations of calcium and inorganic phosphate in the solutions used by these workers were approximately those found in normal serum; but as was pointed out by Robison [1926], this apparent similarity is misleading owing to the absence of the other constituents of plasma, particularly the proteins, which are present in a concentration of 7 % and greatly reduce the ionisation of calcium. In Shipley's solutions as little as 2 % egg-albumin was sufficient to inhibit calcification. To bring about deposition of the bone salt from plasma, therefore, some mechanism for increasing the concentration of calcium or phosphate ions would be required, and such a mechanism is furnished by the bone-phosphatase.

The experiments described in this paper were planned to study the effect of serum-proteins on the calcification of hypertrophic cartilage *in vitro*, both in inorganic solutions and in those containing phosphoric ester.

Experimental.

The method of experiment was similar to that described by Robison *et al.* [1930]. Slices from the heads of the long bones of rachitic rats were immersed for periods of 16 to 20 hours in the sterile experimental solutions at 37° and p_H 7·4. They were then fixed, stained with silver nitrate and cleared in cedarwood oil. The amount of new calcification in the zone of hypertrophic cartilage was judged by examination under low magnification and is shown in the tables by a figure (maximum 10) representing the extent of the deposit, and bars (maximum 5) indicating the density, thus 6≡. Except where otherwise stated each experiment was carried out with bone slices from the same rachitic rat.

Only those modifications in the technique necessitated by addition of protein will be described in greater detail.

Serum-proteins were obtained from normal horse serum by saturation with ammonium sulphate, followed by solution of the precipitate and reprecipitation. The ammonium sulphate precipitate was dialysed in cellophane sacs against hot tap water, distilled water and finally 0·8 % NaCl, and the stock protein solution thus obtained was filtered through Seitz filters and stored at 0° in a sterile bottle fitted with a syphon for withdrawal of samples. The protein content of

[1] Grocers' Company Research Student. The work described in this paper formed part of a thesis approved for the degree of Ph.D. in the University of London.

the solution was estimated by Kjeldahl nitrogen determinations and also by refractometer[1].

The stock protein solution used for most of the experiments quoted contained 0·76 g. N per 100 ml. (≡ 11 % protein), and further, 0·8 g. NaCl, 9·55 mg. Ca, 0·53 mg. Mg and 0·1 mg. P per 100 ml. This was diluted as required with a basal salt solution of such concentration that the final salt contents of the calcifying solutions containing protein and of the protein-free solutions should be the same.

Protein-salt solutions containing the requisite amounts of calcium and inorganic and organic phosphate, and made up in slightly more concentrated form than finally required, were sterilised by filtration through a Seitz filter into a pyrex flask[2].

From the sterile protein-salt solutions the final solutions were prepared in 20 ml. flasks by addition of water, glucose or other constituents as required. The p_H of these solutions was adjusted to 7·35–7·4. Measurements were made with a capillator with correction for protein error and were checked by occasional electrometric measurement.

The sterility of the solution in each flask was tested at the end of the experimental period by incubating a drop on an agar slope. Experiments in which there was serious contamination with consequent fall in p_H were rejected.

The inhibitory effect on calcification in vitro *of protein in various concentrations.*

In the experiment quoted in Table I, bone slices from the same rat were immersed in solutions containing 0, 1·5 and 3 % serum-proteins at various

Table I. *Effect of serum-proteins on calcification* in vitro.

16 hours; 37°; p_H 7·4.

mg. per 100 ml.			Protein g. per 100 ml.		
Ca	P inorg.	P org.	0	1·5	3
			Calcification *in vitro*		
10	4	0	6 ≡	2 =	0
10	6	0	.	6 ≡	7 =
10	4	3	8 ≡	8 ≡	.
10	4	10	.	8 ≡	7 ≡

All the bone slices were from the same rachitic rat.

levels of calcium, inorganic and organic phosphate. In absence of protein there was good calcification in the inorganic solution; 10:4:0[3] but calcification in

[1] For assistance in preparing these solutions, and for the refractometer measurements I am indebted to Dr W. T. J. Morgan.

[2] Estimations of calcium and magnesium were at first made on the non-sterile solutions before filtration, but later it was found that appreciable amounts of Mg and Ca were being extracted from the Seitz asbestos pads. In this way the concentrations of Mg and Ca were being raised by as much as 0·7 mg. Mg and 1 to 1·5 mg. Ca per 100 ml.

Tests showed (*a*) that silica was not extracted simultaneously, and (*b*) that all the soluble Mg and Ca could be extracted by first passing through the pads 50 ml. of a solution similar to the basal salt solution but free from Ca and Mg. This modification of the technique described above was adopted in all further experiments, although the certainty of sterilisation by filtration was thereby somewhat lessened. Only a few of the early experiments in which the Ca and Mg concentrations were raised by filtration have been quoted, and these are marked with an asterisk.

[3] The experimental solutions are referred to in this way throughout the text. Thus, solution 10 : 4 : 3 contains 10 mg. Ca, 4 mg. P as inorganic phosphate and 3 mg. P as phosphoric ester per 100 ml.

this solution was partially inhibited by 3 % protein. In solution 10 : 6 : 0 and in the ester solution 10:4:10 calcification still occurred even in presence of 3 % protein.

Table II. *Effect of serum-proteins on calcification* in vitro.

17 hours; 37°; p_H 7·4.

mg. per 100 ml.			Rat	*a*		*b*		*c*		*d*		*e*	
Ca	P inorg.	P org.	Protein g./100 ml.	0	7	0	7	0	7	0	7	0	7
10	3	0		.	.	1−	.	.	.	.	.	0	.
10	4	0		6≡	.	2=	0	7≡	0	2≡	.	2=	0
10	5	0		.	.	6≡	0	.	2=	.	0	.	0
10	6	0		.	1=	.	4≡	.	5≡	.	0	.	0
10	7	0		.	2=	.	5≡	.	7≡	.	0	.	.
10	8	0		.	5≡	.	.	.	.	.	2≡	.	.

The concentration of protein was raised to 7 % in the experiments quoted in Table II. Considering first the ester-free solutions, it will be seen that the lowest level of Ca × P at which calcification regularly took place was 10 × 6 in presence of protein as compared with 10 × 3 in absence of protein. In one experiment only, out of a large number, was calcification obtained at the lower level 10 × 5 in protein solution (rat *c*). Where the calcifying power of the bones was low, as in rat *d*, a level as high as 10 × 8 was necessary in the protein solutions before calcification occurred.

The inhibitory effect of glucose on calcification in protein-salt solutions.

It is shown elsewhere that glucose has an inhibitory effect on calcification *in vitro* in protein-free solutions [Robison and Rosenheim, 1934]. From the results given in Table III it will be seen that this inhibitory effect was equally marked in 7 % protein solution. Inhibition due to 0·01 % glucose was not demonstrable, but in presence of 0·03 % there was total inhibition of calcification in solution 10 : 7 : 0 and partial inhibition in solution 10 : 9 : 0. In presence of 0·05 or 0·1 % glucose calcification was only twice obtained at a level lower than 11 × 10 in a total of 11 experiments, but no obvious progressive inhibition of calcification was observed with concentrations of glucose greater than 0·1 %.

Table III. *Effect of glucose on calcification* in vitro *in presence of protein.*

Protein 7 g./100 ml.; 18 hours; 37°; p_H 7·4.

	mg. per 100 ml.			Glucose mg. per 100 ml.						
Exp.	Ca	P inorg.	P org.	0	10	30	50	75	100	150
34	10	6	0	4≡	.	.	.	.	0	.
	10	7	0	5≡	.	.	.	.	1=	.
30	10	8	0	2≡	.	.	.	.	0	.
	10	10	0	.	.	.	.	.	0	.
17*	10	7	0	4=	4=	0	0	.	0	.
	10	9	0	7≡	7≡	3≡	0	.	0	.
15*	11	9	0	7≡	.	.	0	.	0	.
	11	10	0	9≡	.	.	4=	.	5≡	.
23*	11	10	0	9≡	.	.	5≡	3≡	2=	2=
	10	12	0	9≡	.	.	.	5≡	3=	6≡

The inhibitory effect of magnesium on calcification in protein-salt solutions.

It has been shown [Shipley and Holt, 1927; Kramer *et al.*, 1927; Robison *et al.*, 1930] that magnesium exerts a definitely inhibitory effect on calcification in inorganic solutions, so that to obtain a given amount of deposit progressively higher levels of the Ca × P product are needed as the magnesium concentration is increased. The two experiments given in Table IV show that the same effect is exerted also in presence of glucose and protein.

Table IV. *Effect of magnesium on calcification* in vitro *in presence of protein and glucose.*

Protein 7 g./100 ml.; glucose 100 mg./100 ml.; 17 hours; 37°; p_H 7·4.

Rat *a*					Rat *b*				
mg. per 100 ml.			mg. Mg per 100 ml.		mg. per 100 ml.			mg. Mg per 100 ml.	
Ca	P inorg.	P org.	1·1	3·2	Ca	P inorg.	P org.	2·6	4·1
11	7	0	2 =	0	10	6	0	0	.
11	8	0	1 =	0	10	7	0	2 =	.
11	9	0	1 =	0	10	8	0	3 =	0
11	10	0	4 ≡	2 =	10	9	0	6 ≡	3 =
11	12	0	7 ≡	6 ≡	10	10	0	.	5 =
					10	12	0	.	8 ≡

It will be seen that with a low magnesium concentration of 1·1 mg. per 100 ml. calcification was obtained in bone slices from rat *a* at the Ca × P level 11 × 7, in presence of 7 % protein and 0·1 % glucose. When the magnesium concentration was increased to 3·2 mg. per 100 ml. no calcification was obtained at a level lower than 11 × 10.

In bone slices from rat *b* calcification was obtained in presence of 7 % protein, 0·1 % glucose and 2·6 mg. Mg per 100 ml. at the exceptionally low Ca × P level of 10 × 7 and in presence of 4·1 mg. Mg, at the level 10 × 9. The inhibition due to increased magnesium concentration is here still apparent, although the bones used for this experiment showed a definitely higher calcifying potentiality than usual, thus further illustrating that variability discussed elsewhere [Rosenheim, 1934].

The beneficial effect of phosphoric ester on calcification in protein-salt solutions.

The effect of sodium glycerophosphate on calcification in presence of 7 % protein was next investigated. Results obtained in glucose-free solutions are given in Table V. They show that there was no calcification at levels lower than 12 × 6 in absence of ester, whereas in presence of glycerophosphate equivalent to 3 mg. P per 100 ml. calcification occurred at the level 12 × 4. In solution 12 : 4 : 10 calcification was more extensive and denser than in solution 12 : 7 : 0.

The results given in Table VI show that the beneficial effect of phosphoric ester was equally marked in solutions containing 0·1 % glucose.

Table V†. *Effect of phosphoric ester on calcification* in vitro *in presence of protein.*

Protein 7 g./100 ml.; 17 hours; 37°; p_H 7·45.

	mg. per 100 ml.		Glycerophosphoric ester mg. organic P per 100 ml.		
Exp.	Ca	P inorg.	0	3	10
5*	12	4	0	0	6≡
	12	6	2–	.	.
7*	12	4	.	4=	9≡
	12	5	0	5≡	7≡
	12	6	3=	.	.
	12	7	6≡	.	.

† It will be noted that the slightly increased Ca and Mg concentrations in the solutions used for the experiments quoted in this and the following table (see footnote 2, p. 700) do not in any way affect the conclusions drawn, as the results in each experiment are on a strictly comparable basis.

Table VI. *Effect of phosphoric ester on calcification* in vitro *in presence of protein and glucose.*

Protein 7 g./100 ml.; glucose 100 mg./100 ml.; 37°; p_H 7·4.

		mg. per 100 ml.		Glycerophosphoric ester mg. organic P per 100 ml.				
Exp.	Time (hours)	Ca	P inorg.	0	0·5	1	3	10
8*	17	12	4	.	.	.	0	3≡
		12	5	0	.	0	0	.
		12	6	0	.	.	.	.
		12	7	2≡	.	.	.	.
9*	17	12	5	.	.	.	0	7≡
		12	6	0	0	0	0	7≡
11*	17	12	6	0	.	.	3≡	5≡
		12	7	0	.	0	2≡	6≡
		12	8	0	.	1=	4≡	.
12*	10	12	8	0	.	3–	4=	8≡
		12	9	4=	4=	7–	7=	8=
14*	17	10	8	0	.	0	5≡	.
		10	9	0	.	0	6≡	.
		10	10	5≡	.	6≡	7≡	.

Calcification in vitro *in the serum of various species.*

Having obtained the above data with regard to calcification *in vitro* in artificial protein-salt solutions, a further series of experiments was carried out with the sera of normal animals.

It was essential to obtain the blood without the use of anaesthetics, at the same time avoiding any condition involving fright or surgical shock, all of which may lead to a rapid increase in the inorganic phosphate of the serum. It was equally important to avoid haemolysis even in slight degree, since this would liberate appreciable amounts of phosphoric ester from the red blood corpuscles.

Serum was obtained from adult rabbits by bleeding from the ear vein and from young rabbits and rats by heart puncture, precautions being taken to maintain sterility. Some difficulty was encountered in obtaining sufficient sterile serum from young rats for the calcification experiment and for the necessary analyses.

The serum was separated from the corpuscles as rapidly as possible to avoid the alteration in inorganic phosphate content which may occur when whole blood stands at room temperature [Martland and Robison, 1926]. The serum was then measured into small sterile tubes, and when required, inorganic phosphate was added in the most concentrated form possible, the dilution of the serum not usually exceeding 0·5 %. The p_H was adjusted to 7·4 with sterile alveolar air or CO_2. In all the experiments quoted, control slices from the same animal were immersed for the same length of time in solution 8:4:0 or 8:5:0 and good calcification was invariably obtained even when the volume of solution was reduced to 2 ml. In small volumes of serum the p_H tended to fall slightly during the experimental period.

In Table VII are shown the results of experiments on the calcification of bone slices of rachitic rats in the serum of normal rats. They are arranged in order of increasing Ca × P product of the serum. Results (1)–(4) in any one experiment were obtained with bone slices from the same animal. The negative result obtained with the serum of one rachitic rat (*R*), in which the Ca × P product was only 28, is included in this table.

The lowest level of the Ca × P product at which there was calcification without addition of extra phosphate was 87 (Exp. 15 (1) and (2)). No calcification occurred in 17 hours in a normal serum of Ca × P level 57 (Exp. 13 (1)), but in samples of the same serum to which inorganic phosphate was added to bring up the levels to 68 and 80 respectively there was slight calcification.

Table VII. *Calcification* in vitro *in rat serum.*

Exp.	Vol. of serum ml.	Time (hours)	mg. per 100 ml. Ca	Inorganic P Serum	Inorganic P Added	Ca × P	Calcification
*R*16	2·5	17	10·3	2·7	.	28	0
13 (1)	2·0	17	9·1	6·3	.	57	0
(2)	,,	,,	,,	,,	1·2	68	<1 –
(3)	,,	,,	,,	,,	2·5	80	3 =
15 (1)	,,	16	9·9	8·8	.	87	3 =
(2)	,,	40	,,	,,	.	,,	3 =
10	2·5	17	9·5	9·6	.	91	8 ≡
9	3·0	16	11·2	8·2	.	92	6 =
7	2·0	18	12·1	8·6	.	104	8 ≣
Y 6	2·0	17	10·9	11·7	.	129	7 ≡

R = Rachitic young rat. *Y* = Normal young rat.

Experiments on the calcification of rat bones in rabbit serum are summarised in Table VIII. The serum-calcium of rabbits is higher than that of rats and subject to considerable fluctuations [Culhane, 1927; Kapsinow and Underhill, 1929] but in the samples used the product Ca × P was generally lower than in rat's serum. A Ca × P level higher than 77 could not be reached without addition of inorganic phosphate, and calcification was not obtained in adult rabbit's serum without such addition, the lowest level for calcification being 87, artificially produced. The inorganic phosphate in the serum of young rabbits is, however, slightly higher than in that of the full-grown animals, and in one such serum with a Ca × P product of 109, slight calcification occurred in 41 hours.

Table VIII. *Calcification* in vitro *in rabbit serum.*

Exp.	Vol. of serum ml.	Time (hours)	mg. per 100 ml. Ca	Inorganic P Serum	Inorganic P Added	Ca × P	Calcification
3 (1)	5	40	15·8	3·0	.	47	0
11 (1)	2	17	17·5	2·84	.	50	0
(2)	,,	,,	,,	,,	.	,,	0
8 (1)	1	18	14·4	3·8	.	55	0
(2)	2	,,	14·0	4·0	.	56	0
2 (1)	5	40	14·0	4·0	.	56	0
(2)	,,	,,	,,	,,	1·0	70	0
1 (1)	6	16	15·0	5·12	.	77	0
(2)	,,	40	,,	,,	.	,,	0
3 (2)	5	19	15·8	3·0	2·5	87	1 −
2 (3)	,,	40	14·0	4·0	2·3	88	2 =
8 (3)	2	18	14·0	4·0	2·3	,,	3 =
11 (3)	,,	17	17·5	2·8	2·5	93	5 ≡
(4)	,,	,,	,,	,,	3·5	110	5 ≡
3 (3)	5	19	15·8	3·0	2·5	130	8 ≡
(4)	,,	,,	,,	,,	8·0	174	10 ≡
Y 4 (1)	2	23	12·5	8·6	.	108	0
(2)	5	,,	,,	,,	.	,,	0
Y 5 (1)	,,	41	13·0	8·4	.	109	1 −
(2)	,,	,,	,,	,,	1·8	134	4 ≡

Y = Young rabbit.

Although samples of rat serum containing 10 mg. Ca and 9 mg. P per 100 ml., and rabbit serum containing 15 mg. Ca and 6 mg. P per 100 ml. have the same Ca × P product of 90 there is no suggestion that the same percentage of calcium is ionised in the serum of these two species or that their calcifying power is the same. The assumption that the calcifying power is directly proportional to the simple product Ca × P is theoretically unjustifiable, so that clearly only a limited significance can be attached to a comparison of solutions and sera on this basis.

The fact that calcification in rat serum is not markedly better than in rabbit serum, and only slightly better than in artificial 7 % horse serum-protein solution, disposes of the objection that in some experiments failure to use homologous serum, or rat serum-proteins, might be affecting the results.

Table IX. *Calcification* in vitro *in human serum.*

Exp.	Serum	Vol. of serum ml.	Time (hours)	mg. per 100 ml. Ca	Inorganic P Serum	Inorganic P Added	Ca × P	Calcification
12	Adult	1	17	9·6	3·9	.	37	0
		2	,,	,,	,,	.	,,	0
		,,	,,	,,	,,	1·1	48	0
		,,	,,	,,	,,	2·3	59	5 =
		,,	,,	,,	,,	3·4	69	5 =
		,,	,,	,,	,,	4·6	81	5 ≡
14	Child 10 years	2	18	10·8	5·3	.	57	0
		,,	,,	,,	,,	0·9	66	1 =
		,,	,,	,,	,,	1·8	76	3 ≡
	Child 4 years	,,	,,	10·1	6·3	.	64	1 =

The results of experiments with the serum of human adults and children are given in Table IX. The levels adequate for calcification were lower than in rat and rabbit serum. Definite calcification occurred when inorganic phosphate was added to raise the Ca × P levels from 37 to 59 and from 57 to 66 respectively.

Calcification occurred without added phosphate in serum from a child of 4 years, the Ca × P product being 64[1].

In general it will be seen that the higher the Ca × P product of the solution bathing the bone slices, the denser and more extensive was the new deposit obtained.

Discussion.

It is known that a considerable proportion of the total calcium of blood is present in non-diffusible form and is probably largely combined with the plasma-proteins. The consequent reduction in the concentration of calcium ions must profoundly affect the calcifying power of the plasma, a conclusion which is fully borne out by the results of the experiments described in this paper. The addition of serum-proteins to the inorganic calcifying solutions rendered very much higher levels of the product Ca × P necessary, in order that calcification of the bone slices should take place *in vitro*.

Since calcification in the body may take place more slowly and under more favourable conditions than in these experiments it may reasonably be expected that somewhat lower levels of the product Ca × P than those here required may suffice for calcification *in vivo*. These experiments, therefore, do not give precise information as to the lowest value of this product which would be effective in the body. They show that under the experimental conditions the sera of very young animals, and sometimes also of older animals, may bring about calcification within 20 hours in the hypertrophic cartilage of rachitic rats, but that with many sera it is necessary to increase the phosphate concentration artificially in order that this result may follow.

Still higher levels of the product Ca × P were required with artificial solutions similar to plasma in their content of inorganic salts, protein and glucose, but here, as in all previous experiments, the necessary Ca × P level was notably reduced if phosphoric ester was added.

The better results obtained with natural as compared with artificial sera may well have been due to the small amount of phosphoric ester present in the former; but other constituents of serum not included in the artificial solutions may also have influenced the results.

Summary.

1. The effect of serum-proteins in different concentrations on the calcification *in vitro* of hypertrophic cartilage has been investigated.

2. In presence of horse serum-proteins in 7 % concentration the lowest Ca × P level at which calcification regularly took place in solutions containing no phosphoric ester was 10 × 6 as compared with 10 × 3 in absence of protein.

3. The inhibitory effect of glucose on calcification observed in non-protein solutions was apparent also in presence of 7 % protein and was additional to the effect of the latter, so that in salt solutions containing 0·1 % glucose and 7 % protein calcification was not usually obtained at a level lower than 11 × 10.

4. The inhibitory effect of magnesium on calcification previously observed in non-protein solutions was apparent also in presence of protein.

[1] Shipley *et al.* [1926] have stated that they obtained calcification in bone slices immersed in children's serum of Ca × P product 50 to 54.

5. In presence of glycerophosphoric ester calcification in protein-salt solutions took place at lower levels of the Ca × P product than in its absence. Thus in bone slices from the same rat calcification was obtained in solution 12 : 4 : 3, but not in solution 12 : 6 : 0.

6. Calcification *in vitro* was obtained in the serum of adult rats and rabbits at a Ca × P level of 80–90, and in human serum at a level of about 60. In the serum as drawn with precautions to avoid alteration in inorganic phosphate concentration the requisite level of the Ca × P product was rarely reached, so that the addition of inorganic phosphate was necessary in order that calcification should take place under the experimental conditions.

It is a pleasure to acknowledge my indebtedness to Prof. R. Robison and to thank him for his help and encouragement throughout the course of this work.

REFERENCES.

Culhane (1927). *Biochem. J.* **21**, 1015.
Kapsinow and Underhill (1929). *J. Biol. Chem.* **82**, 377.
Kramer, Shelling and Orent (1927). *Bull. Johns Hopkins Hosp.* **41**, 426.
Martland and Robison (1926). *Biochem. J.* **20**, 847.
Robison (1926). *Biochem. J.* **20**, 388.
—— Macleod and Rosenheim (1930). *Biochem. J.* **24**, 1927.
—— and Rosenheim (1934). *Biochem. J.* **28**, 684.
—— and Soames (1930). *Biochem. J.* **24**, 1922.
Rosenheim (1934). *Biochem. J.* **28**, 708.
Shipley and Holt (1927). *Bull. Johns Hopkins Hosp.* **41**, 1.
—— Kramer and Howland (1926). *Biochem. J.* **20**, 379.

XCVII. VARIABILITY IN THE ACTIVITY OF THE CALCIFYING MECHANISM IN THE BONES OF RACHITIC RATS.

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It was noted by Robison *et al.* [1930] that the calcifying power attributed to the second mechanism in the hypertrophic cartilage varied considerably in different rachitic rats, even in those of the same age and litter. The variation was manifested by differences in the minimum level of the Ca × P product required for calcification *in vitro* in inorganic solutions and also in the extent of the deposit obtained at higher levels.

Experiments bearing on the relation of such variation to the rate of growth and the condition of the rachitic animals are presented in this paper.

For the first group of experiments litters were selected in which the rats showed unusually varied growth over a 3 to 4-week period on McCollum 3143 diet, and the possible connection of this variation in growth rate with the calcifying power of the cartilage was investigated. The litter-mates were killed on the same day, and tibia slices from each rat were immersed in 20 ml. of the specified calcifying solutions, one slice per flask. The flasks containing the slices were kept at 37° for 20 hours and rotated continuously. The p_H in all the flasks remained constant at 7·4 throughout each experiment.

The growth curves of the rats are given in Fig. 1 and a summary of the experiments in Table I. In the marks assigned to the calcification the figures, with maximum 10, show the approximate extent of the new deposits, and the bars, with maximum 5, indicate the density. The depth of the uncalcified zone in the control slices varied widely, and the figures show the proportion of this zone which became calcified and not the absolute amount of deposit.

The results show that the second mechanism activity developed in the hypertrophic cartilage for the most part varied directly with the amount of growth of the rat during the period on rachitogenic diet.

The figures given for the inorganic solutions (Exps. 1–5) show the variation in the amount of calcification due to the second calcifying mechanism alone. Those given for the ester solutions (Exp. 2) illustrate in an equally striking manner the variation in the calcification produced by the combined mechanisms under conditions which probably approached more closely to those existing *in vivo*.

In inorganic solution calcification at the low level[2] 8 : 2 : 0 was obtained in the case of two of the rats showing the most growth over the period on diet, while in the solutions 8 : 3 : 0 and 8 : 4 : 0 calcification was more general but

[1] Grocers' Company Research Student. The work described in this paper formed part of a thesis approved for the degree of Ph.D. in the University of London.

[2] The experimental solutions are referred to in this way throughout the text. Thus solution 8 : 5 : 3 contains 8 mg. Ca, 5 mg. P as inorganic phosphate and 3 mg. P as organic phosphate per 100 ml. basal salt solution.

decreased in amount with decreasing rate of growth. In solution 8 : 5 : 0 differences were less pronounced.

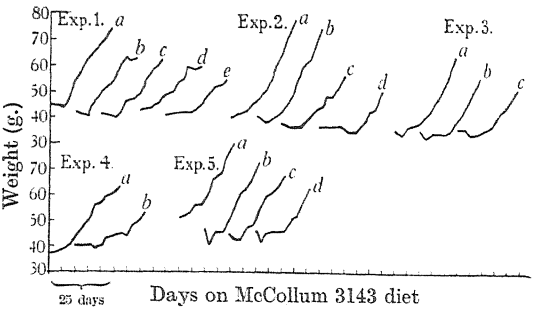

Fig. 1.

Table I.

Exp.	Period on diet (days)	Litter	Rat	Wt. at 21 days* g.	Increase in wt. on diet g.	mg. per 100 ml.	Inorganic solutions: Ca 8	Inorganic solutions: P inorg. 2	Inorganic solutions: P org. 0	Ester solutions: Ca 8	Ester solutions: P inorg. 2	Ester solutions: P org. 1
							8	3	0	8	2	2
							8	4	0	8	2	3
							8	5	0			
1	23	2384	*a* ♂	45	29		0	2=	7=	7≡	.	.	.
			b ♀	42	21		0	<1−	4≡	5≡	.	.	.
			c ♀	41	21		0	<1−	4≡	5≡	.	.	.
			d ♀	43	17		0	0	3≡	5≡	.	.	.
			e ♀	41	14		0	0	2≡	5≡	.	.	.
2	25	2661	*a* ♂	40	38		2=	7≡	7≡	.	5≡	7≡	8≡
			b ♂	41	34		0	4=	5≡	.	3=	5≡	7≡
			c ♀	38	19		0	3=	6≡	.	1−	4≡	6≡
			d ♂	37	14		0	1=	4≡	.	0	3=	3≡
3	21	2869	*a* ♀	36	29		1=	7≡	8≡	.	.	.	.
			b ♀	36	21		0	6≡	8≡	.	.	.	.
			c ♀	37	16		0	1=	5≡	.	.	.	.
4	27	2720	*a* ♀	37	26		0	0	1=	5≡	.	.	.
			b ♀	40	13		0	0	0	5≡	.	.	.
5	21	2675	*a* ♂	52	29		0	3≡	4≡	.	.	.	.
			b ♀	47	26		0	4≡	5≡	.	.	.	.
			c ♂	45	23		0	3≡	5≡	.	.	.	.
			d ♂	46	17		0	3=	5≡	.	.	.	.

* All the litters were 21 days old when placed on the rachitogenic diet.

The relation of calcification to growth rate is not apparent in the case of Litter 2675, Exp. 5. It may be noted that these rats were exceptionally well grown at the beginning of the dietary period.

As regularly observed in previous experiments, the amount of new calcification in the slices from any one rat showed a marked gradation in passing from lower to higher levels of the Ca × P product. The use of such material, that is bone slices taken from the same animal, for observing the effect of various factors on the calcifying mechanisms is thus justified.

The favourable effect on calcification of phosphoric ester even in low concentration is again emphasised by the results of Exp. 2.

In another series of experiments quoted in Table II, three or four rats selected from different litters were killed on the same day after different periods of 1 to 6 weeks on McCollum 3143 diet.

Table II.

Exp.	Rat	Days on diet	mg. per 100 ml.	Inorganic solutions				Ester solutions		
			Ca	8	8	8	8	8	8	8
			P inorg.	3	4	5	6	3	4	3
			P org.	0	0	0	0	3	3	10
1	1 ♀	7		.	10 ≡	.	.	.	.	10 ≡
	2 ♀	10		.	10 ≡	.	.	.	.	10 ≡
	3 ♀	28		.	<1 –	.	.	.	.	7 ≡
	4 ♀	37		.	1 =	.	.	.	.	2 ≡
2	1 ♂	7		.	10 ≡	10 ≡	10 ≡	10 ≡	10 ≡	.
	2 ♀	17		.	2 =	3 ≡	3 ≡	4 ≡	5 ≡	.
	3 ♀	30		.	0	<1 =	<1 =	4 ≡	5 ≡	.
3	1 ♂	15		.	3 =	6 =	.	5 ≡	.	10 ≡
	2 ♂	17		.	2 =	6 ≡	.	6 ≡	.	8 ≡
	3 ♂	28		.	<1 –	<1 =	.	6 ≡	.	6 ≡
	4 ♀	40		.	<1 ≡	<1 ≡	.	4 ≡	.	4 ≡
4	1 ♂	28		2 =	7 ≡	8 ≡	.	.	.	10 ≡
	2 ♀	42		<1 –	3 ≡	7 ≡	.	.	.	8 ≡
	3 ♀	56		<1 –	<1 –	6 ≡	.	.	.	7 ≡

The results show that the calcifying power of the cartilage decreases with increasing periods on a rachitogenic diet.

The position of the deposit in these bone slices is a point of considerable interest. In the bones of the young rats which had been less than 2 weeks on the diet (Exp. 1, rats 1 and 2; Exp. 2, rat 1) the whole of the narrow zone of hypertrophic cartilage was apparently in the optimum condition for calcification. Deposition *in vitro* extended from the "line" to the shaft, and the slices calcified *in vitro* resembled bone slices from normal animals of the same age (Plate II, Figs. 2 and 3). In bone slices from the rats which had grown well for 3–4 weeks on a rachitogenic diet, deposition *in vitro* had the appearance familiar in the "line test," that of a band of calcification separated from the diaphysis by a zone of disorganised and less readily calcifiable tissue.

This band of cartilage calcifiable in inorganic solution became narrower as the period on diet increased, until after 4 weeks or more there were large areas of uneroded hypertrophic cartilage which did not become calcified, even in solution 8 : 6 : 0.

It would seem that the calcifying power of the second mechanism is greatest where the cells have just attained full hypertrophy, decreasing after this stage has been passed. A similar conclusion was reached by Niven and Robison in experiments with the cartilage of embryonic rabbit femora [quoted by Robison, 1932]. The same general statement may also be made for the combined calcifying power of the phosphatase and second mechanisms, although the area of cartilage calcified *in vitro* was invariably more extensive in presence of ester than in the corresponding inorganic solutions.

The formation of a calcified line in healing rickets is probably due to the maximum calcifying power of the dual mechanism in this region.

The validity of the argument based on the results shown in Tables I and II may be criticised on the grounds that the observed variations in calcifying power do not in every case run parallel either with the growth rate or with the length of time on the rachitogenic diet. These results do, however, furnish strong evidence that the calcifying power of the cartilage in a rachitic rat varies with the condition of the animal; and this conclusion is supported by the general results of experiments carried out in this laboratory over many years.

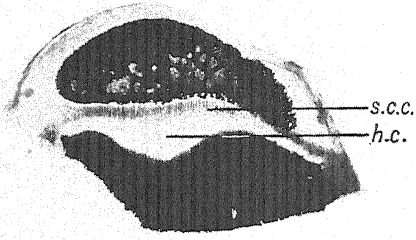

Fig. 1.

Fig. 4.

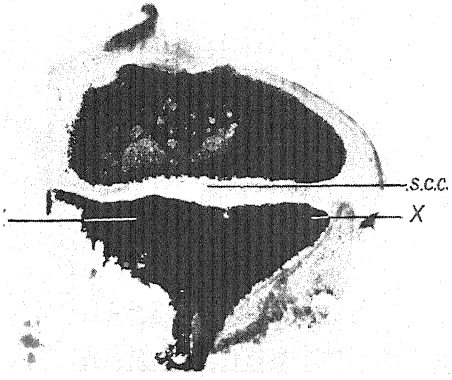

Fig. 2.

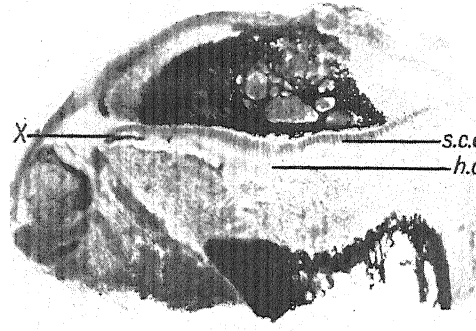

Fig. 5.

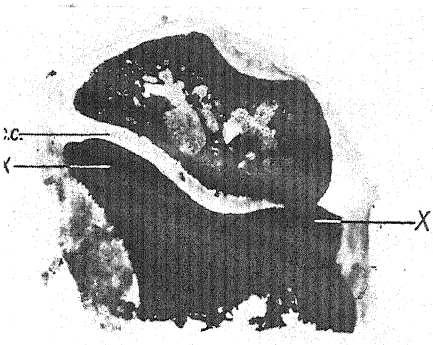

Fig. 3.

Fig. 6.

Whether this variation is connected with deficiency in some specific factor, and whether it is due to the same cause as the variation in the growth rate, cannot at present be decided. The experimental diet (McCollum 3143) was notably deficient in two nutritional requirements, inorganic phosphate and vitamin D. Coward *et al.* [1932] state that "the reserves of vitamin D in the young rat vary in different individuals and in different litters," basing their conclusion on the variation in the growth of these animals on a vitamin D-deficient, but otherwise adequate, basal diet. The results of the experiments described in this paper are not inconsistent with the view that the observed variations in the calcifying power of the cartilage may, like those in the growth rate, be a result of differences in the vitamin reserves of the animals. Although it is held on good grounds that changes in the composition of the blood are primarily responsible for the deficient calcification in rickets, it is difficult to avoid the conclusion that changes in the calcifying power of the cartilage may also play a part in the story.

SUMMARY.

The calcifying power of the cartilage in the bones of rachitic rats varies with the rate of growth of the animals over a 3 to 4-week period on a rachitogenic diet and decreases as the period on diet is increased.

I should like to thank Prof. R. Robison for much help and valuable criticism.

REFERENCES.

Coward, Key and Morgan (1932). *Biochem. J.* **26**, 1505.
Robison (1932). Herter lectures (New York).
—— Macleod and Rosenheim (1930). *Biochem. J.* **24**, 1927.

DESCRIPTION OF FIGURES IN PLATE II.

Tibia slices stained by v. Kossa's silver nitrate method.

X = calcification *in vitro* in the hypertrophic cartilage zone.
h.c. = hypertrophic cartilage.
s.c.c. = small-celled cartilage.

Figs. 1–3. Table II, Exp. 1, Rat 2. 10 days on a rachitogenic diet.

Figs. 4–6. Table II, Exp. 1, Rat 3. 28 days on a rachitogenic diet.

Fig. 1. Control slice. Note the narrow zone of uncalcified hypertrophic cartilage.

Fig. 2. Slice after immersion for 20 hours in the inorganic solution 8 : 4 : 0 at 37° and p_H 7·4.

Fig. 3. Slice after immersion for 20 hours in the ester solution 8 : 3 : 10. Note the dense band of new calcification extending to the diaphysis in this slice and in that shown in Fig. 2.

Fig. 4. Control slice. Note the breadth of the uncalcified zone.

Fig. 5. Tibia slice after 20 hours in solution 8 : 4 : 0. The very small amount of new calcification (<1) obtained *in vitro* indicates the decreased calcifying power of the hypertrophic cartilage. Note the position of the deposit on the "line."

Fig. 6. Tibia slice from the same rat as (5) after 20 hours in solution 8 : 3 : 10. Calcification is denser and more extensive than in absence of phosphoric ester.

XCVIII. THE CALCIFICATION *IN VITRO* OF KIDNEY, LUNG AND AORTA.

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ALTHOUGH calcification in the animal body is normally confined to hypertrophic cartilage and osteoid, in certain abnormal or pathological conditions deposits of calcium salts may also be formed in other tissues, among which are the kidney, the lung and the arteries. Some of these conditions, for example hypervitaminosis D, are associated with exceptionally high concentrations of blood-calcium or phosphate, suggesting that the plasma has become by some agency supersaturated with calcium salts. In others degenerative changes in the tissues themselves precede, and may possibly be the primary cause of, calcification.

Without attempting to reproduce *in vitro* all the abnormal conditions concerned it seemed of importance to discover whether the calcification of such tissues could be induced in our experimental solutions, and if so, in what respects the process would differ from that taking place in hypertrophic cartilage and osteoid. Experiments of this kind, though carried out with the tissues of normal animals, might yet throw light on abnormal calcification and, by differentiation, on the normal process of calcification in the skeleton.

Two questions of particular interest were: (*a*) whether the phosphatase which occurs in some of these tissues is at all responsible for their calcification, and (*b*) whether these tissues possess in any degree properties akin to those which constitute the second mechanism of cartilage.

EXPERIMENTAL.

The technique was similar to that employed for experiments on the calcification *in vitro* of cartilage and bone. Calcifying solutions of similar composition were used [Robison and Rosenheim, 1934]. They are referred to as solution 8 : 6 : 10, 8 : 6 : 0, *etc.*, according to their content of calcium, inorganic P and organic P respectively in mg. per 100 ml. of basal salt solution. The organic phosphorus was in the form of α-glycerophosphoric ester.

Our experiments were confined to the kidney, lung and aorta. The tissues were obtained from normal rats and rabbits of various ages and in a few cases from young rachitic rats. They were excised as rapidly as possible, with precautions to avoid bacterial contamination, and small pieces were immersed in the warm calcifying solutions at p_H 7·4 and left at 37° for various periods. Subsequently these pieces were fixed in 10 % neutral formalin, washed, dehydrated and embedded in paraffin. Serial sections were cut, stained with silver nitrate and counterstained. A black deposit of metallic silver formed in the

[1] Grocers' Company Research Student. The work described in this paper formed part of a thesis submitted by A. H. Rosenheim and approved for the degree of Ph.D. in the University of London.

sections on exposure to light was accepted as evidence of the presence of solid phosphate or carbonato-phosphate of calcium, that is, of tissue calcification. The deposits obtained were never so heavy as to be readily apparent before sectioning and staining.

Pieces of each tissue, fixed without immersion in the calcifying solutions, were sectioned and stained as controls.

In some experiments small pieces of the same kidney, lung or aorta were left for different lengths of time in samples of the same calcifying solution, each piece in a separate flask. In other experiments the pieces of each tissue were immersed for the same length of time but in a number of solutions differing in their content of calcium, inorganic phosphate and phosphoric ester.

The results obtained show that calcification of kidney, lung and aorta *in vitro* can be realised under suitable experimental conditions, but that such calcification takes place much more slowly than that of hypertrophic cartilage and is much more erratic in occurrence and distribution.

No attempt has been made to group the results of individual experiments together, since these were often irregular, one piece of tissue possibly showing calcification in 4 days while another piece of the same tissue might fail to calcify in a longer period or in a solution of higher Ca × P level.

The positive and negative results obtained with rat's kidney and lung are collected in Table I and those obtained with the aortae of rats and rabbits in Table II.

From Table I it will be seen that calcification occurred with some regularity in pieces of rat's kidney and lung immersed in solutions containing phosphoric ester, but that the deposits were not generally formed in less than 2 days. In absence of phosphoric ester calcification was only once obtained in kidney and not once in lung, although a large number of experiments were performed with highly supersaturated inorganic solutions and the period of immersion was extended to 4 days.

Table I. *Calcification of kidney and lung* in vitro.

		Inorganic solutions		Solutions containing phosphoric ester			
mg. per 100 ml.	Ca	8	8	8	8	8	8
	P inorg.	5	6	4	5	6	4
	P org.	0	0	10	10	10	30
Tissue	Days						
Kidney	1	– – – –	– – – –	– –	– –	– – –	+
		.	– – – –	.	.	– – –	– –
	2	– –	+	+	–	+	+ +
		.	– –	.	.	.	.
	3	–	–	.	.	.	.
	4	–	–	.	.	.	.
Lung	1	– –	– –	.	–	–	–
	2	–	–	.	–	+ +	+
	3	.	.	.	.	.	+
	4	–	–	.	+	+	.

Each sign in Tables I and II refers to a separate piece of tissue, calcification *in vitro* being indicated by + and absence of calcification by –.

In the aortae both of rats and rabbits, however, calcification did occur in the inorganic solutions, although not invariably and never in less than 3 days (Table II). These solutions were very highly supersaturated and would certainly have produced regular and extensive calcification in cartilage within 10–16 hours.

Table II. *Calcification of aorta* in vitro.

		Inorganic solutions			Solutions containing phosphoric ester			
mg. per 100 ml.	Ca	8	8	8	8	8	8	8
	P inorg.	4	5	6	4	5	6	4
	P org.	0	0	0	10	10	10	30
Species	Days							
Young rat	1	.	−	.	−	.	.	−
	2	.	− −	−	−	.	−	−
	3	.	− −	−	−	.	−	−
	4	.	−	− −	− −	− −	−	.
	5	.	−	− −	−	−	+	.
		.	.	.	.	.	−	.
Full-grown rat	1	.	−	−	−	−	−	.
	2	.	−	.	−	−	−	.
	3	.	−	+	−	.	−	.
	4	.	+	− − −	−	+ + +	+	−
		.	−	.	.	− − −	−	.
	5	.	+	+ +	.	+	+ +	.
		.	.	− − −	.	−	− − −	.
	6	.	−	− −	.	+	+	.
		.	.	.	.	.	− −	.
	7	.	.	+	.	−	.	.
	10	.	.	.	.	.	+	.
Young rabbit	3	.	−	.	.	−	.	.
	4	−	−	.	.	.	.	.
	5	− −	+ +	+	.	− −	−	.
		.	−	.	.	.	.	.
	6	.	+	.	.	−	.	.
Full-grown rabbit	2	.	.	− − (− −)	.	.	−	.
	3	+	− −	− −	.	.	−	.
	4	.	.	+	.	.	.	.
	5	+	+ (+ +)	+	.	−	−	−
		.	− − (− −)	−	.	.	.	.
	6	.	.	+	.	.	.	.
	8	.	+	+	.	.	−	.

Signs in brackets refer to lengths of aorta treated with acetone or desiccated (see text).

In rat's aorta calcification was somewhat more frequent and was denser in presence of phosphoric ester but no such effect was observed in rabbit's aorta. This result was expected, since it has been shown that the former tissue contains phosphatase [Macfarlane *et al.*, 1934], while the latter is devoid of the enzyme [Kay, 1928]. From the results quoted the presence of glycerophosphoric ester would indeed appear to have had some inhibitory effect on the calcification of rabbit's aorta, and this too is reasonable, since the unhydrolysed ester must compete with the inorganic phosphate for calcium ions.

It was noted that the deposits formed in the aorta whether in presence or absence of ester became more extensive and denser as the period of immersion was prolonged and also that the aortae of full-grown rats and rabbits became calcified more readily than those of younger animals. On the other hand, there was no evidence that the age or condition of the animal influenced the calcification of kidney and lung *in vitro*. These tissues were taken from young rachitic rats, young normal rats and adult rats, and positive and negative results were distributed fairly evenly among the three groups.

Effect of acetone and of desiccation on the calcifiability of the rabbit's aorta.

It has previously been shown that the second calcifying mechanism of hypertrophic cartilage is seriously damaged by treatment of the cartilage with acetone or alcohol, or by desiccation [Robison *et al.*, 1930]. Experiments were, therefore, performed to discover whether similar treatment would affect the calcifiability of rabbit's aorta which possesses no phosphatase. The aortae of three full-grown rabbits were used. One portion of each aorta was immersed directly in the calcifying solution while another portion was first soaked in acetone for 22 hours and a third dried over sulphuric acid *in vacuo*. In two experiments solution 8 : 5 : 0 was used and the period of immersion was 5 days. In the third experiment the solution was 8 : 6 : 0 and the period only 2 days. Calcification was obtained in one of the 5-day experiments in the two lengths of aorta which had been treated with acetone and desiccated respectively (Plate III, Fig. 4), but not in the control piece. There was no calcification in any part of the aortae used for the other two experiments. It is clear, therefore, that the calcifiability of the aorta is not diminished but rather enhanced by acetone treatment and desiccation, and that the qualities leading to the calcification of this tissue are markedly different in this respect from the second mechanism of hypertrophic cartilage.

Description of the deposits.

(*a*) *Kidney.* Calcification *in vitro* was usually confined to a narrow zone of cortex beginning a few cell layers from the exterior, though in some cases the extreme peripheral zone was the most densely calcified. The deposits were finely granular and extended over the basement membranes of the tubules and often throughout the tubule cells. The nuclei of these cells also appeared to be calcified (Plate III, Fig. 1). Deposits were frequently observed in the glomeruli and on the glomerular capsules. The distribution of the deposits formed in the rat's kidney *in vitro* bears some resemblance to that of the calcification in the same tissue described by Brand and Holtz [1929] and produced by overdosage with irradiated ergosterol.

Although half kidneys or transverse slices were used in these experiments so that at least one cut surface of the medulla was always exposed to the solution, only slight traces of calcification were observed in this region. In this connection it is of interest that the cortex has been shown by Kay [1926] to possess considerably greater phosphatase activity than the medulla.

(*b*) *Lung.* The deposits obtained in lung were not localised in any special part of the organ. There was diffuse granular deposition, denser in some places than in others and extending irregularly throughout the sections examined. It was not confined to the cells lining the alveoli but appeared also in the alveolar spaces. There was fairly heavy calcification in the walls of the bronchioles (Plate III, Fig. 2).

(*c*) *Aorta.* Calcification *in vitro* in different regions of the same aorta was very varied in amount, being usually densest in the ascending aorta and aortic arch (Plate III, Fig. 3). Its distribution thus corresponded with that of the calcified areas observed in the aorta in experimental hypervitaminosis D in the rat and the rabbit as described by Duguid [1930] and Vanderveer [1931] respectively.

Calcification commenced most often on the outer side of the media or in the adjacent layers of the adventitia; sometimes, however, it occurred in the middle or inner layer of the media, while the outer layers remained free from deposit. Occasionally the calcified zone extended in places almost through the entire

thickness of the vessel wall though adjoining parts of the wall might be free from deposit (Plate IV, Fig. 8).

The elastic fibres in the broad medial coat are the most prominent feature seen in the stained transverse sections of the aortic arch and dorsal aorta of rats and rabbits. The intima is not well developed. The bands of deposit stretched along and between the elastic laminae (Plate IV, Figs. 5 and 7) and the fine granules of calcium salt very often appeared to have been formed on the fibres themselves (Plate IV, Fig. 8). Relatively large rounded aggregates definitely located at intervals along the fibres were sometimes seen (Plate IV, Fig. 6). Wenzel [1928] has observed the deposition of calcium salts in granular form on the elastic fibres in experimental hypervitaminosis D; while deposits in the media, between the elastic laminae, have been described by Kreitmair and Hintzelmann [1928].

Various experiments on calcification of the aorta.

Certain further experiments which were essentially of a preliminary nature may be briefly referred to here.

1. Although there was little evidence of marked degeneration in the tissues except after the most prolonged periods of immersion, less obvious physico-chemical changes must have been taking place from the time of excision. That such degenerative changes might be essential for calcification seemed the more possible in view of the relative slowness with which calcification set in and its sporadic appearance.

In one of the experiments undertaken to investigate this point, an excised rat aorta was kept 3 days at 37° in a solution of Ca × P product too low to produce calcification. Pieces of the tissue were then transferred to the more concentrated solutions 8 : 6 : 0 and 8 : 5 : 10 and left for further periods of 1, 2 or 4 days. Calcification did not occur in less than 4 days after transference of the tissues to the latter solutions. Such degeneration of the tissue as may have occurred during the preliminary period did not, therefore, serve to produce more rapid calcification than usual.

In other experiments sections of aorta were cut on the freezing microtome and alternate sections were stained for fat and calcium salts. Although the stainable fat was found to increase in amount with increasing periods of immersion at 37° there seemed to be no connection between the occurrence and location of fatty degeneration and the occurrence of calcification.

2. With the usual technique the pieces of aorta immersed in the experimental solutions remained in a highly contracted condition very different from that of the aorta *in vivo*. In a few experiments an attempt was made to maintain part of the vessel wall in a distended state. A piece of the aorta was slit open and stretched between the ground ends of two lengths of glass tubing, which were clamped together. A few ml. of 8 : 6 : 10 solution were forced through the tissue drop by drop at a pressure of about 160 mm. Hg over a period of 4 days. During this time solution and tissue were maintained at 37°. Calcification, however, did not take place.

3. Calcification of certain tissues is known to occur as a result of injury. A length of aorta was, therefore, purposely injured by scraping with a sterile scalpel or by pinching with forceps before immersion in the calcifying solutions. Such injury did not lead to increased calcification *in vitro*.

4. Since permeability of the aorta wall may be a factor limiting the rate of calcification and since testicular extract has been shown to exert a marked effect in increasing tissue permeability [McClean, 1931], an experiment was

carried out in which 0·2 ml. of this extract (for which we are indebted to Drs McClean and Morgan) was injected into the media of a rabbit's aorta before excision. There was no evidence of calcification in this aorta after 3 days in 8 : 5 : 0 solution.

Discussion.

If the results of these experiments are to be applied to the problems of pathological calcification it is clear that great caution must be used. In the body deposits of calcium salts are frequently found in abnormal or necrotic tissues whereas the calcification which was obtained *in vitro* occurred in tissues taken for the most part from normal animals. The deposits obtained in the arteries *in vitro* would, indeed, appear to bear less resemblance to those occurring in such conditions as Mönckeberg's sclerosis or atheroma (associated with muscular and fatty degeneration respectively) than to those formed in the early stages of experimental hypervitaminosis D in which tissue degeneration is relatively slight and appears to be of secondary nature [Wenzel, 1928; Vanderveer, 1931]. In the lung and kidney also the calcification *in vitro* was not obviously related to any marked degenerative changes; though doubtless some tissue degeneration must have occurred during the prolonged period of immersion.

There are grounds for assuming that in hypervitaminosis D the condition of supersaturation of the circulating fluid with calcium salts is a determining factor in tissue calcification, as it is in our experimental solutions. In necrotic or degenerate tissues factors of a different type are introduced.

In connection with the etiology of arterial disease it is of some interest that in our experimental solutions calcification occurred in isolated pieces of aorta subjected to no mechanical stresses and not distended by the force of the blood stream.

The experiments described in this paper have shown that in a medium supersaturated with calcium salts, calcification of certain tissues other than hypertrophic cartilage and osteoid may occur without participation of phosphatase, although if the tissue contains this enzyme and phosphoric ester is present the likelihood of deposition will be increased. These results do not suggest that the tissue phosphatases are essential factors in pathological calcification[1]. At least one of the tissues whose calcification was studied, the aorta, possesses qualities which can provoke deposition of calcium salts in the tissue from supersaturated solutions before spontaneous precipitation occurs in the solution itself. By this empirical criterion we should recognise that the aorta possesses the second mechanism, but the slow and erratic formation of the deposits indicates a very low degree of activity. It is not proved that the mechanism in the aorta is identical with that in cartilage. There is, indeed, some evidence to the contrary, for example, in the different effect of acetone on these two tissues. In the aorta the qualities which favour calcification are perhaps associated with the surface of the fibres. It is not unlikely that similar forces at fibrillar interfaces may also assist deposition in cartilage, even if the high activity of the second mechanism in this tissue should ultimately prove to be due to an enzyme complex, as we have suggested elsewhere [Robison and Rosenheim, 1934].

The results of these experiments again emphasise the very special position occupied by hypertrophic cartilage and osteoid with respect to the dual calcifying mechanism. They show also that certain other tissues possess qualities

[1] In heterotopic ossification, in which true osteoid tissue is developed in abnormal locations, phosphatase probably plays the same part as in normal bone formation [Huggins, 1931].

which, although unable to effect their calcification under normal conditions, may do so if the calcium-phosphate level in the blood is abnormally raised. Whether such qualities become further developed as a result of tissue degeneration must be left for future investigation.

SUMMARY.

1. Calcification of the kidney, lung and aorta excised from normal animals has been realised *in vitro* by immersion of the tissues in experimental solutions of similar composition to those used for calcification *in vitro* of hypertrophic cartilage.

2. Calcification of these tissues took place much more slowly than that of hypertrophic cartilage, and especially in the aorta, was erratic in occurrence and distribution.

3. Calcification occurred with some regularity in pieces of rat's kidney and lung immersed in solutions containing phosphoric ester, but not usually in less than 2 days. In absence of phosphoric ester calcification was only once obtained in kidney and never in lung.

4. Calcification occurred, though not invariably, in aortae both of rats and rabbits immersed in very highly supersaturated inorganic solutions for periods of 3 to 8 days.

5. The deposits formed in the kidney, lung and aorta *in vitro* had some resemblance in appearance and distribution to those described as occurring in the same tissues *in vivo* in experimental hypervitaminosis D.

REFERENCES.

Brand and Holtz (1929). *Z. physiol. Chem.* **185**, 217.
Duguid (1930). *J. Path. Bact.* **33**, 696.
Huggins (1931). *Biochem. J.* **25**, 728.
Kay (1926). *Biochem. J.* **20**, 791.
—— (1928). *Biochem. J.* **22**, 855.
Kreitmair and Hintzelmann (1928). *Arch. exp. Path. Pharm.* **137**, 203.
Macfarlane, Patterson and Robison (1934). *Biochem. J.* **28**, 720.
McClean (1931). *J. Path. Bact.* **34**, 459.
Robison, Macleod and Rosenheim (1930). *Biochem. J.* **24**, 1927.
—— and Rosenheim (1934). *Biochem. J.* **28**, 684.
Vanderveer (1931). *Arch. Path.* **12**, 941.
Wenzel (1928). *Arch. exp. Path. Pharm.* **137**, 215.

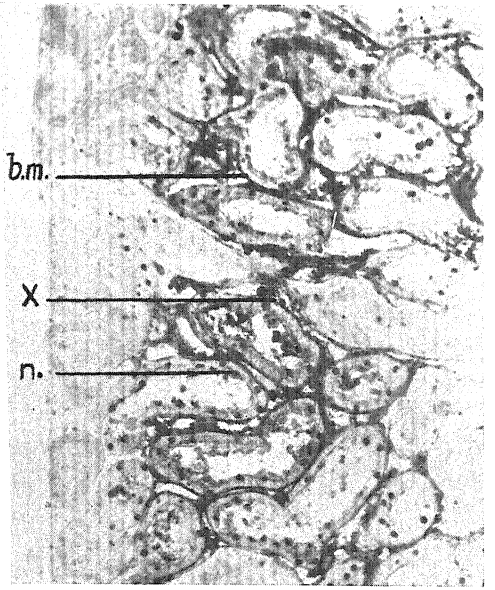


Fig. 1.

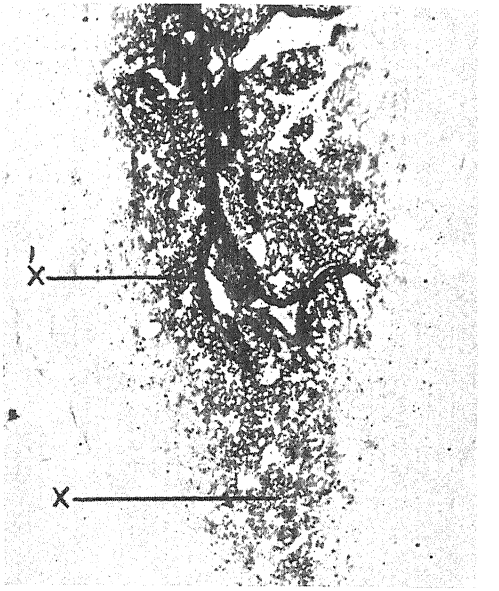
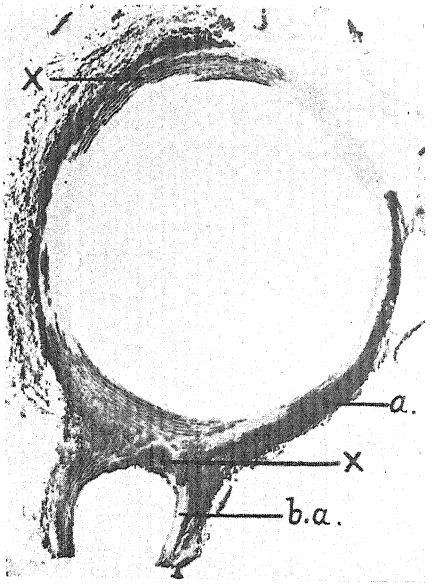


Fig. 2.



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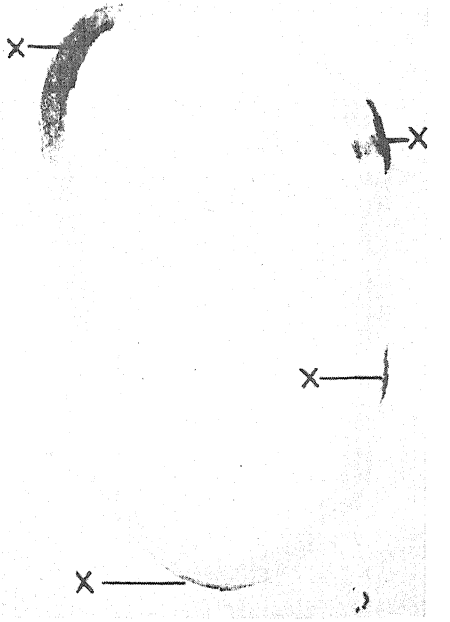


Fig. 4.

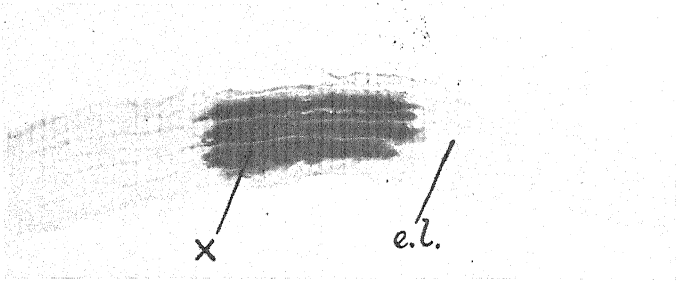
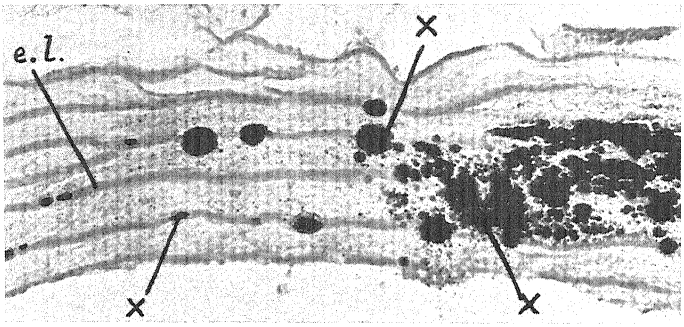


Fig. 5.



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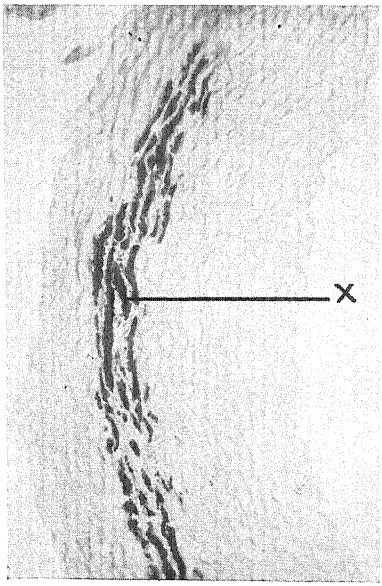

Fig. 7.

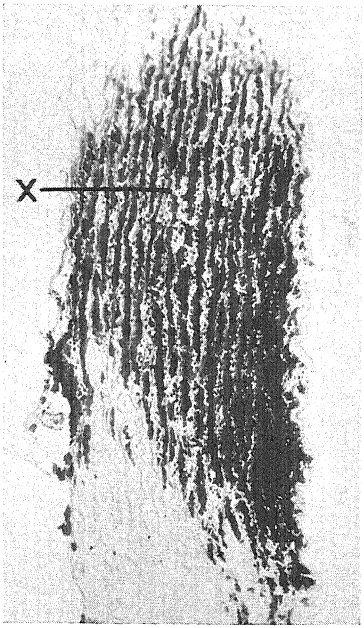

Fig. 8.

DESCRIPTION OF FIGURES IN PLATES III AND IV.

Sections of kidney, lung and aorta, cut after immersion of the tissues in various calcifying solutions for the periods specified. The sections were treated with silver nitrate to show the tissue calcification (*X*) which occurred *in vitro*.

a. = aorta.
b.a. = branch artery.
b.m. = basement membrane of tubule cell.
e.l. = elastic lamina.
n. = nucleus of tubule cell.

PLATE III.

Fig. 1. Cortex of the kidney of a full-grown rat. 2 days in solution 8 : 4 : 30. Note the calcification in the basement membrane of the tubules and in the nuclei of the tubule cells. (Mag. × 250.)

Fig. 2. Lung of the same rat. 4 days in solution 8 : 5 : 10. (Mag. × 27.)

Fig. 3. Transverse section of the aortic arch of a full-grown rat. 4 days in solution 8 : 5 : 10. Note the heavy calcification of the adventitia and media in the main and branch arteries. (Mag. × 22.)

Fig. 4. Aorta of rabbit. The tissue was soaked for 22 hours in acetone and subsequently immersed for 5 days in solution 8 : 5 : 0. The section illustrates the patchy distribution of calcification *in vitro* noted also in tissues not subjected to acetone treatment. (Mag. × 20.)

PLATE IV.

Fig. 5. Thoracic aorta of a full-grown rat. 3 days in solution 8 : 6 : 0. Note the deposit apparently located in the bands of muscle and connective tissue between the elastic laminae. (Mag. × 140.)

Fig. 6. Part of the same aorta as in Fig. 3. 5 days in solution 8 : 6 : 0. Note the rounded aggregates apparently formed in and on the elastic laminae. (Mag. × 350.)

Fig. 7. Aorta of full-grown rabbit. 3 days in solution 8 : 4 : 0. Slight calcification was obtained in the outer half of the media. The deposit is apparently located between the elastic laminae. (Mag. × 140.)

Fig. 8. Aorta of full-grown rabbit. 5 days in solution 8 : 5 : 0. Note the heavy calcification extending almost through the entire thickness of the artery wall. The deposit is chiefly between the laminae, but where it is densest it would seem to overlap them. In places fine granules are visible on the fibres between the bands of deposit. (Mag. × 140.)

XCIX. THE PHOSPHATASE ACTIVITY OF ANIMAL TISSUES.

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(*Received March 9th, 1934.*)

The presence of a highly active phosphatase in bone and ossifying cartilage forms the basis of a conception of the process of calcification in these tissues for which much evidence has been accumulated [Robison, 1923]. That calcification does not normally occur in all tissues which contain phosphatase is not necessarily prejudicial to this conception since the animal organism makes wide use of phosphoric esters of varied type and it is not surprising that many of its tissues should contain enzymes by which the synthesis and hydrolysis of these esters can be accomplished. We were, however, interested in the possibility that the presence of phosphatase may be correlated with the calcification which occurs in some of these tissues in certain abnormal bodily states.

The distribution and behaviour of the tissue phosphatases have been the subject of many investigations, which have recently been reviewed by Kay [1932], who discusses the limitations of the experimental methods employed. It has long been recognised that these phosphatases are not all identical, while Akamatsu and his co-workers have shown that more than one type may be present in the same tissue [Akasawa, 1929; Kurata, 1931]. The present experiments were concerned solely with the distribution of the phosphomonoesterase of high optimum p_H which occurs in bone. They were intended to supplement the results already obtained on the relative activities of the tissues of different animal species. It was desired also to eliminate the possibility that a negative or low phosphatase activity reported for any tissue was due simply to lack of magnesium in the tissue or tissue extract. The pronounced effect of magnesium on phosphatase derived from different sources has been described by Erdtman [1927; 1928] and by Jenner and Kay [1931]; the latter concluded that magnesium acts as an accelerator rather than as a coenzyme.

In these experiments we have made a more extended trial of a method which had been found valuable in a study of phosphatase development in rudimentary bones and minute explants of embryonic tissue [Fell and Robison, 1929; 1930]. A number of pieces of each tissue, roughly 1·0–4·0 mg. dry weight, were cut as nearly uniform in size and shape as possible, placed on cover slips and rapidly dried in an evacuated desiccator. The tissue fragments were weighed on a microbalance and placed separately in stoppered tubes of 5 ml. capacity to which 2 ml. distilled water and a small drop of chloroform were added. After standing 24 hours at room temperature, 2 ml. of a 0·2*M* solution of sodium β-glycerophosphate (p_H 8·9) were added, together with a further drop of chloroform; if necessary the p_H was adjusted to 8·6. From one of the duplicate tests carried out on each tissue examined, a sample was then removed for estimation of inorganic phosphate. This 0-hour value, which rarely exceeded 0·004 mg. P

per ml., represented the sum of the inorganic phosphate present in the glycerophosphate solution *plus* that dissolved out of the tissue and was deducted from the final value obtained. The tubes were placed in an incubator for 48 hours at 25° or 24 hours at 37°; at the end of this time the p_H was checked, and suitable samples were removed for estimation of the phosphate by the Briggs method. When the tests were carried out in presence of magnesium this was added as $MgSO_4$, in the glycerophosphate solution, so that the final concentration of Mg was 0·003*M*. This concentration was chosen as the most suitable after preliminary tests on a wide range of tissues. It falls within the limits of optimum concentration found by Jenner and Kay [1931]. No added buffers were used in these experiments since their presence is not without effect on phosphatase activity. Sodium glycerophosphate itself has considerable buffering powers at p_H 8·6; and in more than 90 % of the tests the p_H did not fall below 8·5. Any tests in which the final p_H was lower than 8·4 were rejected. The fall in p_H occurred chiefly in the tests with liver, lung, spleen and muscle and was presumably due largely to glycogenolysis. Since the p_H-activity curve of phosphatase is very steep it may be well to state that differences in the final p_H did not account for the observed variation in the activity of duplicate samples.

The majority of the tests were carried out at 25° for 48 hours, the remainder at 37° for 24 hours. The former conditions usually gave somewhat higher values and were considered preferable owing to the greater stability of the enzyme at the lower temperature.

As a result of many hundreds of tests certain comments may be made on this method.

1. It is specially applicable to investigations in which only minute amounts of tissue are available, as in young or small animals, or in which it is desired to study the localisation of the enzyme in particular zones of the tissue.

2. It gives a direct relationship between the activity and the weight of solid matter in the tissue; the dry weight of tissues with variable water content can be determined with greater accuracy than the wet weight and may provide a more suitable basis for comparison.

3. A large number of tissues from the same animal can be conveniently examined.

4. Although in certain tissues reasonably good agreement was obtained between duplicate samples, in others a wide variation was found, indicating uneven distribution of the enzyme. A limited number of tests may therefore fail to give a fair indication of the average phosphatase activity of a tissue.

5. Since duplicate determinations can only be carried out on separate pieces of tissue it is impossible to check the accuracy of individual values.

6. The assumption that no loss of phosphatase activity occurs during drying may not be equally correct for all tissues, and is difficult to verify exactly in the case of tissues with variable phosphatase content. There was, however, no indication of any serious loss of activity.

7. Because of the variation in individual values, the method is not the most suitable for studying the exact effect of p_H and other factors on the phosphatase activity of different tissues.

As these comments indicate, the method possesses definite advantages for certain purposes but does not replace the usual method in which the moist tissues are ground with chloroform water.

The average values of A/W obtained for tissues of animals of different species are shown in Table I, those for tissues of rats of different ages in Table II. The tissues include some for which no data were previously available. In general

Table I. *Phosphatase activity (A/W) of mammalian tissues.* (*Average values.*)

A = inorganic phosphate (mg. P) liberated from 0·1 *M* Na β-glycerophosphate in 48 hours at p_H 8·6 and 25°.
W = weight of tissues (mg.).

Final conc. of $MgSO_4$ added ...	None						0·003 *M*					
Animal	Rat	Rabbit	Guinea-pig	Mouse	Cat	Dog	Rat	Rabbit	Guinea-pig	Mouse	Cat	Dog
No. of animals examined ...	3	3	1	2	1	1	3	3	1	2	1	1
Age of animals (weeks) ...	4	6–8	4	12	9	5	4	6–8	4	12	9	5
Tissue												
Femur epiphysis	0·25	0·22	—	—	—	—	0·60	0·56	—	—	—	—
Costochondral junction	0·28	0·53	—	—	0·88	0·73	1·10	1·10	—	—	3·18	2·40
Rib cartilage	<0·01	<0·01	<0·01	<0·02	0	0	0·02	<0·01	—	<0·02	0·01	0·03
Trachea	0·03	<0·01	<0·01	<0·02	0·03	0·02	0·12	0·02	0·06	0·02	0·14	0·27
Intestinal mucosa	—	0·43	2·1	0·67	3·0	2·6	—	1·13	2·42	6·77	7·75	11·8
Kidney cortex	0·38	0·16	0·32	0·66	0·46	0·41	0·55	0·22	0·79	2·70	1·50	1·4
Bladder	0·07	0·06	0·07	0·13	0·06	0·03	0·16	0·16	0·13	0·50	0·35	0·08
Aorta	0·09	<0·01	<0·01	<0·01	0	0	0·38	<0·01	<0·01	0?	0?	0
Dorsal aorta	0·09	<0·01	—	0	—	0	0·38	<0·01	—	0	—	0
Cardiac muscle	—	0·01	0·03	0·05	0·01	<0·01	—	0·02	0·05	0·09	0·04	0·02
Skeletal muscle	<0·01	—	0·02	0·03*	0·01	—	—	—	—	0·10*	0·01	0·01
Lung	0·17	0·07	0·10	0·06	0·11	0·05	0·61	0·20	0·25	0·10	0·21	0·28
Liver	0·03	0·06	0·02	0·03	0·02	0·06	0·05	0·12	0·04	0·06	0·07	0·26
Spleen	—	0·07	—	0·03	0·03	0·09	—	0·21	—	0·15	—	0·36
Pancreas	—	0·03	—	0·03	0·02	0·11	—	0·06	—	0·04	0·03	0·36
Thyroid	0·16	0·02	0·03	0·12	0·02	0·01	0·41	0·05	0·13	0·30	0·07	0·04
Adrenal	0·09	0·03	0·33	0·02	0·06	0·03	0·26	0·05	0·73	0·05	0·29	0·11
Thymus	—	0·06	—	0·03	—	0·11	—	0·07	—	0·10	—	0·48

* Intercostal muscle.

Table II. *Phosphatase activity (A/W) of tissues of rats of different ages. (Average values.)*

A = inorganic phosphate (mg. P) liberated from 0·1 *M* Na β-glycerophosphate in 24 hours at p_H 8·6 and 37°.
W = weight of dried tissue (mg.).

Final conc. of $MgSO_4$ added...	None			0·003 *M*		
Age of rat	1 day	4 weeks	6 weeks	1 day	4 weeks	6 weeks
No. of rats examined ...	2	3	2	2	3	2
Tissue						
Femur epiphysis	0·23	0·21	0·18	1·23	0·48	0·57
,, junction	—	0·48	0·33	—	1·04	0·79
,, shaft	0·36	0·07	0·07	1·45	0·27	0·16
Rib bone	—	—	0·14	—	0·31	0·30
Costochondral junction	0·20	0·17	0·18	0·77	0·40	0·52
Tooth point	0·36	0·09	0·06	1·04	0·25	0·13
,, root		0·41	0·25		1·18	0·59
Membrane bone	0·40	0·08	0·10	1·26	0·57	0·23
Rib cartilage	<0·01	<0·01	<0·01	0·02	0·01	0·01
Trachea	0·03	0·03	0·04	0·25	0·16	0·24
Intestinal mucosa	0·67	—	0·80	2·94	2·06	2·42
Kidney cortex	0·16	—	0·34	0·37	0·38	0·68
,, medulla		—	0·04		0·11	0·15
Bladder	0·06	0·08	0·05	0·20	0·19	0·27
Aorta	0·08	0·02	0·14	0·11	0·45	0·86
Cardiac muscle	—	—	0·06	—	0·10	0·10
Skeletal muscle	<0·01	<0·01	<0·01	0·08	0·01	0·02
Lung	0·29	0·12	0·10	0·42	0·24	1·08
Liver	0·02	—	0·02	0·05	0·04	0·03
Spleen	0·05	—	0·02	0·20	0·07	0·09
Brain	0·06	—	0·04	0·10	0·06	0·07
Thyroid	0·05	0·04	0·04	0·38	0·21	0·42
Adrenal	0·04	0·03	0·06	0·28	0·20	0·23
Thymus	0·02	—	0·02	0·07	0·06	0·08

the relative activities are in agreement with those obtained by other methods. In nearly all cases the activity was decidedly increased in presence of magnesium. High values are shown for intestinal mucosa, kidney cortex, bones and teeth; with regard to the last two, it should be remembered that a large proportion (50–66 %) of the dry tissue fragment consisted of inorganic material, so that the A/W of the organic portion was actually much higher than the value shown. Rib cartilage and skeletal muscle were almost inactive in all species examined, even in the presence of magnesium, but cardiac muscle had a slight but definite activity. Most of the remaining tissues gave intermediate values, rather variable from one species to another. The definite activity of the bladder in all species may be noted [*cf.* Huggins, 1931] and also that of the trachea in the rat, cat and dog. Calcification of the tracheal cartilage occurs not infrequently *in vivo*, especially in old age; and the presence of phosphatase may seem significant when it is recalled that in the rudimentary bone the appearance and increasing activity of this enzyme runs parallel with the hypertrophy of the cartilage cells.

The examination of the aorta was carried out with special interest, in view of the frequency with which this tissue becomes calcified in certain abnormal states. Among the species examined, the aorta of the rat alone showed definite phosphatase activity; and in this respect the contrast between the rabbit and the rat is remarkable. Calcification of the aorta has been frequently observed *in vivo* in both rat and rabbit, and calcification has also been induced *in vitro*

in the excised aortae of both animals [Rosenheim and Robison, 1934]. While therefore the phosphatase present in the rat aorta may affect in some degree the incidence or extent of such calcification, it would seem that the enzyme cannot be the crucial factor in this process.

SUMMARY.

A method of estimation of the phosphatase activity of tissue fragments is described in which the activity is related to the dry weight of the tissue. The application of this method is discussed.

The values obtained by this method for the phosphatase activity of various tissues in presence and absence of magnesium are given for the rat, rabbit, guinea-pig, mouse, cat and dog. The results generally confirm those previously obtained.

The bladder shows definite phosphatase activity in all species examined; the trachea also showed some activity in most species.

The aorta of the rat was exceptional in possessing a definite phosphatase activity, while in the other species this tissue was practically devoid of the enzyme.

REFERENCES.

Akasawa (1929). *J. Biochem.* **11**, 143.
Erdtman (1927). *Z. physiol. Chem.* **172**, 182.
—— (1928). *Z. physiol. Chem.* **177**, 211.
Fell and Robison (1929). *Biochem. J.* **23**, 767.
—— —— (1930). *Biochem. J.* **24**, 1905.
Huggins (1931). *Biochem. J.* **25**, 728.
Jenner and Kay (1931). *J. Biol. Chem.* **93**, 733.
Kay (1932). *Physiol. Rev.* **12**, 385.
Kurata (1931). *J. Biochem.* **14**, 25.
Robison (1923). *Biochem. J.* **17**, 287.
Rosenheim and Robison (1934). *Biochem. J.* **28**, 98.

C. THE EFFECTS OF THE INJECTION OF CERTAIN SALTS ON THE CALCIUM, MAGNESIUM AND INORGANIC PHOSPHORUS OF THE SERUM OF THE RABBIT.

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(*Received March 1st, 1934.*)

Brookfield [1933] showed that in the serum of the rabbit there was an inverse relationship between calcium and magnesium, a direct relationship between magnesium and inorganic phosphorus and an inverse relationship between calcium and inorganic phosphorus, the latter of which had been previously observed by Dupré and Semeonoff [1931] and Bourne and Campbell [1932]. The rises and falls in the concentrations of the three ions in the serum were correlated with their relative amounts in the diet. The relationships seemed worthy of further study under other experimental conditions, in order to determine whether the effects observed with diets varying in their mineral contents were of more general significance, and accordingly another series of experiments has been carried out.

Solutions of salts containing Ca^{++}, Mg^{++} and $PO^{\equiv}_4$ ions have been injected subcutaneously into rabbits and their effects on the calcium, magnesium and inorganic phosphorus of the serum observed.

Experimental.

Experiments were made on fully grown male rabbits maintained on a diet of cabbage and oats, which (with one exception) had fasted for about 18 hours. Calcium gluconate, laevulate and lactate, magnesium sulphate and lactate and disodium hydrogen phosphate were the salts employed, while control experiments were carried out with sodium chloride and sodium sulphate. The salts, dissolved in the minimum volume of water, were injected subcutaneously into the flank. 5 ml. of blood were withdrawn from the lateral vessel of the ear immediately before the injection and on three separate occasions afterwards. The effects were observed over periods varying from $1\frac{1}{2}$ to 6 hours. The methods of estimating calcium, magnesium and inorganic phosphorus were the same as those used in the dietary experiments [Brookfield, 1933].

The influence of two factors called for control before the results of the experiments could be interpreted; firstly, the effects of four small haemorrhages recurring at relatively short intervals and secondly, the effects of the injection of a solution of an indifferent salt.

1. *The effects of bleeding.* (*a*) *Successive small haemorrhages.* 5 ml. of blood were removed from a rabbit four times at half-hourly intervals; 9 days later the same procedure was repeated, but at hourly intervals and after a further 22 days the animal was bled again, this time at 2-hourly intervals. In the

first experiment the haemoglobin percentage was unaltered at the fourth bleeding; in the other two experiments a fall of 8 % was noted. The serum-calcium, magnesium and inorganic phosphorus values are recorded in Table I.

Table I. *Serum-calcium, magnesium and inorganic phosphorus. Small haemorrhages at short intervals.*

Rabbit 20. Weight 2010 g. 4 bleedings of 5 ml. on each occasion.

Calcium mg./100 ml.	Magnesium mg./100 ml.	Inorganic phosphorus mg./100 ml.
½-hourly intervals		
16·55	1·952	3·152
15·70	1·694	2·626
15·15	1·597	2·551
14·95	1·590	2·716
1-hourly intervals		
13·29	2·886	4·382
13·29	2·886	4·382
12·94	2·794	4·762
12·72	2·749	4·794
2-hourly intervals		
13·53	3·615	3·750
13·53	3·542	3·936
13·90	3·357	4·521
13·67	3·204	4·654

It will be seen that the magnesium value tends to fall, but at a very slow rate. The calcium figures show variable results, depending on the time interval. The four bleedings at half-hourly intervals have caused a fall of 1·6 mg. (10 %). Hourly bleedings cause a slighter fall (4 %) and 2-hourly bleedings none. A fall in the inorganic phosphorus followed by a rise after one hour results from bleeding at intervals of half an hour, while with hourly or 2-hourly bleedings the inorganic phosphorus rises.

(*b*) *Progressive changes during a single haemorrhage.* The rapidity with which the fall in the serum-calcium takes place is illustrated by the results recorded in Table II.

Table II. *Serum-calcium and magnesium values during bleeding. Blood taken in two samples of 5 ml. each.*

Rabbit no.	Time of bleeding	Calcium mg./100 ml.	Magnesium mg./100 ml.
19	11.45–12.15	15·28	1·722
	12.15–12.45	14·44	1·694
21	4.30–4.45	15·73	1·895
	4.45–5.0	15·35	1·886
19	10.45–11.0	12·60	1·907
	11.0–11.10	12·25	1·896
20	11.15–11.25	14·35	1·963
	11.25–11.30	14·25	1·951

Each animal was submitted to a single haemorrhage and the blood collected in two successive 5 ml. samples. In one experiment where the time of bleeding was rather prolonged, a fall of 0·8 mg. (6 %) was noted. The effects of haemorrhages on the serum-calcium of rabbits have been referred to by several writers. A decrease of 10 % or more has been reported when observations have been made

at a short interval after the haemorrhage by Stransky [1915], Clark [1920], Stewart and Percival [1927] and Culhane [1927]. Seven hours after bleeding, Moritz [1925] found a slight and inconstant decrease in the serum-calcium value. A fall in the magnesium also was noted by Stransky. These results show that the variations in the concentrations of calcium, magnesium and inorganic phosphorus caused by bleeding are so slight as to have little or no significance in the interpretation of the findings in the injection experiments, which will now be discussed.

2. *The effects of the injection of an indifferent salt.* Sodium chloride was injected in an amount somewhat in excess of those of the other salts employed. The results are recorded in Table III.

Table III. *Injection of sodium chloride. Serum-calcium, magnesium and inorganic phosphorus values.*

Rabbit 24. Weight 2300 g. 5 ml. bleedings. Fasting.

Time after injection (hours)	Calcium mg./100 ml.	Magnesium mg./100 ml.	Inorganic phosphorus mg./100 ml.
0	13·29	2·786	3·820
0·36 g. NaCl (0·0062 g. equivalent per kg. in 9 ml. water injected subcutaneously).			
¼	13·29	2·806	4·154
1	12·67	2·726	3·434
4	13·06	2·557	3·701

There is a slight fall in all three constituents after one hour with a tendency on the part of the calcium and inorganic phosphorus to return to their initial values after 4 hours. These changes are unimportant in comparison with the results which follow, and they show that probably any effect observed in the injection of salts containing Ca^{++}, Mg^{++}, or $PO^{=}_{4}$ was to be attributed to these ions themselves.

3. *The injection of solutions containing the Ca^{++}, Mg^{++}, $PO^{=}_{4}$ and $SO^{=}_{4}$ ions.* An attempt was made, in each case, to introduce an amount of salt sufficient to cause a marked effect on the several blood-constituents under review without producing untoward symptoms. The success achieved was only partial. No symptoms were noted after the injection of the calcium salts. Slight ataxia of the hind-limbs resulted from the injection of magnesium sulphate and lactate. Definitely toxic effects were observed however with disodium phosphate and sodium sulphate. In both cases there was some degree of collapse after the injection. The animal injected with disodium phosphate showed marked muscular twitching but ultimately recovered. The animal which received sodium sulphate was found dead 2 days later. No urine was passed during the period of any of the experiments except by the rabbit which received sodium sulphate. None of the animals suffered from diarrhoea.

Results.

For purposes of comparison the amounts of the salts have been expressed as g. equivalents per kg. body weight. The results of the injections of the calcium salts are set out in Table IV, of the magnesium salts in Table V, of sodium sulphate in Table VI and of disodium phosphate in Table VII.

1. *The interrelations of calcium and magnesium.* In each of the 4 experiments where calcium salts have been injected approximately equivalent amounts of calcium have been given. A moderate increase in the serum-calcium has been the result amounting on the average to 3·0 mg. or 23 % of the initial value.

The rise to the maximum takes place within one hour and is followed by an almost equally rapid fall. The rise in the serum-calcium is accompanied by a progressive fall in the serum-magnesium. In the case of the injection of calcium gluconate, there may be a slow return towards normal at 6 hours. The average fall amounts to 1·0 mg. or 37 % of the initial value though it should be noted that in 3 of the experiments (Table IV) the fall appears still to be continuing at the time of the final observation.

Table IV. *Injection of calcium salts. Serum-calcium, magnesium and inorganic phosphorus values.*

	Salt injected	Time after injection (hours)	Calcium mg./100 ml.	Magnesium mg./100 ml.	Inorganic phosphorus mg./100 ml.
Rabbit 19. 2520 g. Fasting	Gluconate (0·6 g.≡0·0028 g. equiv. Ca/kg.)	0	13·17	2·389	3·720
		½	16·13	1·896	3·861
		2	14·91	1·590	4·106
		6	14·32	1·748	4·152
Rabbit 23. 2000 g. Fasting	Gluconate (0·75 g.≡0·0035 g. equiv. Ca/kg.)	0	13·29	3·091	4·826
		½	16·59	2·567	4·700
		1	16·75	2·097	4·609
		1½	17·77	1·896	4·700
Rabbit 23. 2200 g. Fasting	Laevulate (0·6 g.≡0·0039 g. equiv. Ca/kg.)	0	12·27	3·044	4·252
		½	15·19	2·635	3·990
		1¼	14·57	2·136	—
		2¼	13·48	1·860	—
Rabbit 23. 2170 g. Fasting	Lactate (0·46 g.≡0·003 g. equiv. Ca/kg.)	0	14·46	2·332	2·977
		½	17·02	1·902	3·106
		1	14·97	1·620	2·927
		2	14·82	1·544	3·247

Though the magnesium salts have been injected in amounts equivalent to those of calcium the effects have been considerably greater. The rise in the serum-magnesium averages 5·6 mg. over the initial value, as compared with a rise of 3·0 mg. in the calcium value caused by injections of corresponding amounts of calcium salts. This rise takes place rapidly but is more sustained than in the case of calcium, and it is clear that either the rate of absorption of magnesium salts is much less rapid than that of calcium salts, or alternatively and more probably

Table V. *Injection of magnesium salts. Serum-calcium, magnesium and inorganic phosphorus values.*

	Salt injected	Time after injection (hours)	Calcium mg./100 ml.	Magnesium mg./100 ml.	Inorganic phosphorus mg./100 ml.
Rabbit 19. 2680 g. Fed with cabbage and oats	Sulphate (0·37 g.≡0·003 g. equiv. Mg/kg.)	0	14·69	2·109	2·779
		¾	11·32	8·078	2·080
		1¼	10·74	8·258	1·890
		4¾	13·31	3·634	3·086
Rabbit 25. 2750 g. Fasting	Lactate (0·37 g.≡0·0031 g. equiv. Mg/kg.)	0	12·62	2·348	3·682
		¾	11·85	6·726	3·262
		1¾	10·54	7·466	3·147
		5	10·61	5·833	3·608

that the removal of excess magnesium from blood is much less readily accomplished. Where magnesium sulphate has been injected (Table V) the serum-magnesium is approaching the normal value after 5 hours, but at the same interval after the injection of the equivalent amount of magnesium lactate (Table V) the serum-magnesium is nearly 3 times greater than the initial value.

These increases in the magnesium concentration have resulted in a marked fall in the calcium value in every experiment. This fall in calcium has been more rapid and greater in extent (4·0 mg. or 27 % of the initial value), following the injection of magnesium sulphate (Table V). The calcium value in this experiment is approaching the initial level after 6 hours. Following the injection of magnesium lactate the serum-magnesium continues at a high level for a considerable time and there is a correspondingly prolonged depression of the serum-calcium, which after 5 hours is still at the low level of 10·6 mg.

The inverse relationship found to exist between calcium and magnesium in the dietary experiments is therefore borne out by the results of these subcutaneous injections of calcium and magnesium salts.

Previous observations of the effects of the injection of calcium and magnesium salts on the calcium and magnesium content of serum are fragmentary and in some cases conflicting. Salvesen *et al.* [1924, 2] found that the magnesium content of the blood of dogs was depressed by the intravenous injection of calcium chloride. Stransky [1915] and Meneghetti [1927] observed a fall in the serum-calcium of rabbits after the injection of magnesium sulphate, the latter worker also showing that the diffusible calcium was increased. A number of observers have shown that the injection of magnesium salts leads to an increase in the calcium excretion and *vice versa*. Thus Mendel and Benedict [1909] found that the injection of calcium chloride into dogs and rabbits led to loss of magnesium, while the injection of magnesium sulphate and chloride increased the excretion of calcium. Greenwald and Gross [1925] noted a temporary increase in the excretion of magnesium in dogs injected with calcium chloride. After introducing metallic magnesium under the skin of rabbits, Reding and Slosse [1923] noted a considerable increase in the output of calcium, and loss of calcium was also observed by Schiff [1920] and by Schiff and Stransky [1920] in infants injected with magnesium sulphate. While a fall in the serum-calcium, accompanied by an increased excretion of calcium, was noted by Pribl [1929] in rabbits after the injection of magnesium sulphate, a rise in the serum-calcium value with calcium retention following the injection of magnesium lactate was reported. Condorelli's [1926] findings are also at variance with those recorded above, in that he observed a considerable elevation of the serum-magnesium following the injection of calcium lactate into rabbits.

2. *The effects of the sulphate ion.* One possible complicating effect in the experiment with magnesium sulphate is the presence of the sulphate ion, for the fall in the calcium value was less when magnesium was introduced as the lactate. Since the injection of phosphate depresses the serum-calcium it was possible that the injection of sulphate might have had a similar effect, and this was controlled in an experiment in which sodium sulphate was injected in amount equivalent to twice that of the magnesium sulphate used. Table VI shows that this has

Table VI. *Injection of sodium sulphate (1·0 g. Na_2SO_4, $10H_2O \equiv 0{\cdot}006$ g. equiv. Na/kg.). Serum-calcium, magnesium and inorganic phosphorus values.*

Rabbit 23. Weight 2500 g. Fasting.

Time after injection (hours)	Calcium mg./100 ml.	Magnesium mg./100 ml.	Inorganic phosphorus mg./100 ml.
0	14·34	2·629	4·700
½	13·52	2·629	3·536
1¾	12·20	2·505	3·106
4	11·20	2·557	3·572

resulted in a considerable fall in the serum-calcium but one different in character from that caused by the injection of magnesium sulphate. The fall is much more gradual, and although still continuing at the time of the last observation, 4 hours after the injection, is less than that produced by half the equivalent amount of magnesium sulphate (Table V). It is of interest that Stransky [1915] found a much increased calcium excretion after the injection of sodium sulphate into a rabbit. Table VI shows that while sodium sulphate has no effect on the serum-magnesium, the serum-inorganic phosphorus is depressed by 1·6 mg. (34 %) to rise again after one and three quarter hours, when it moves in a direction opposite to that of calcium.

3. *The interrelations of calcium and inorganic phosphorus.* The changes in the serum-inorganic phosphorus after the injection of the three calcium salts (Table IV) are so slight that it appears that injected calcium has no direct effect on the inorganic phosphorus. When, however, disodium phosphate is injected there is an immediate fall in the serum-calcium (Table VII). In this experiment the phos-

Table VII. *Injection of disodium hydrogen phosphate (0·57 g. Na_2HPO_4, $12H_2O \equiv$ 0·0047 g. equiv. P/kg.). Serum-calcium, magnesium and inorganic phosphorus values.*

Rabbit 19. Weight 2300 g. 5 ml. bleedings. Fasting.

Time after injection (hours)	Calcium mg./100 ml.	Magnesium mg./100 ml.	Inorganic phosphorus mg./100 ml.
0	12·97	2·529	4·121
1	11·39	2·308	10·01
2	10·74	2·423	10·51

phate was injected in amount equivalent to 1·5 times the amounts of the salts used for the calcium injections. The rise in the serum-inorganic phosphorus was rapid and relatively prolonged: at the end of one hour it was 5·9 mg. (140 %) above the initial value, and at 2 hours was at a slightly higher level. At the same interval after the injection, the serum-calcium had fallen by 2·2 mg. or 17 %. Samples were unobtainable after 2 hours owing to the collapsed state of the animal.

From these results it is plain that the inverse relationship between the calcium and inorganic phosphorus of serum observed in the dietary experiments is only partially maintained when calcium or phosphate is injected. The results of the injection of phosphate are in accord with the dietary experiments inasmuch as the serum-calcium falls as the inorganic phosphorus rises. Several previous workers have reported a depression of the serum-calcium on injecting phosphate. Binger [1917] produced a fall of the serum-calcium to 6 mg./100 ml. with the development of tetany by injecting dogs intravenously with disodium phosphate. Tisdall [1922] repeated his experiments with a similar effect, while Salvesen *et al.* [1924, 1] obtained the same results when phosphates were administered orally. The calcium thus displaced from the serum is apparently largely excreted since Greenwald and Gross [1925] found a rise in the calcium excretion in dogs after the injection of neutral sodium phosphate, while Boyd *et al.* [1930] observed, in addition to a fall in the serum-calcium, an increased urinary excretion of calcium after injecting sodium glycerophosphate into the same animals.

In the present investigation injection of calcium has produced no definite fall in the serum-inorganic phosphorus, so that in this respect there is no effect analogous to the inverse relationship of the dietary experiments. The results of other workers are somewhat variable. Bomskov [1930] found that a fall in the

inorganic phosphorus followed the injection of calcium gluconate into rabbits. Salvesen *et al.* [1924, 2] and Collip [1926], using dogs, reported a small rise in the inorganic phosphorus value on injecting calcium chloride. Condorelli [1926] found a greater rise in the rabbit injected with calcium lactate. Greville [1931] found the inorganic phosphorus of the serum of two rabbits slightly elevated after the intravenous injection of calcium laevulate, while Apitzsch [1931] observed that the serum-inorganic phosphorus of human subjects was raised after the oral administration of calcium lactate. Thus the balance of evidence suggests that there is no disappearance of phosphorus from the blood, though it is noteworthy that Greenwald and Gross [1925] found an increased excretion of phosphorus in the dog after the injection of calcium chloride.

4. *The interrelations of magnesium and inorganic phosphorus.* Following the injection of magnesium (Table V) there is a fall in the serum-inorganic phosphorus amounting, after the injection of the sulphate to 0·9 mg. (32 %) and after that of the lactate to 0·5 mg. (15 %). The somewhat greater effect of magnesium sulphate is possibly accounted for by the synergic action of the sulphate ion which itself lowers the serum-inorganic phosphorus (Table VI). It will be seen that the injection of phosphate (Table VII) caused no appreciable change in the serum-magnesium, just as the injection of calcium caused no effect on the serum-inorganic phosphorus. These results are not in accord with the direct relationship between magnesium and inorganic phosphorus observed in the dietary experiments.

Discussion.

The injection experiments differ from the earlier dietary experiments in two important respects which may account to some extent for some of the divergences in the results of the two series. Firstly, whereas in the injection experiments salts were introduced subcutaneously and rapidly absorbed into the circulation, in the dietary experiments much smaller amounts of the relevant ion were slowly absorbed from the intestine over a period occupying 2 hours or more. Secondly, while two ions only were concerned in the injection experiments, a number of ions were being absorbed simultaneously in the dietary experiments, as well as other constituents which may themselves exercise important effects.

These differences do not appear to be of sufficient importance materially to affect the relationship existing between the calcium and magnesium of serum, which in both series of experiments is inverse. It is noteworthy that the effects of the injections have not been as drastic as might be anticipated. In the dietary experiments the rises and falls in the serum-calcium corresponded with similar rises and falls in the serum-magnesium, whereas in the injection experiments the greater and more rapid increase in the concentration of the one ion in the blood has produced a fall in the other hardly greater than those seen in strictly physiological conditions. It is evident that there exist mechanisms whereby the organism can resist attempts to lower a blood constituent to an undesirable level.

When the relations of calcium and magnesium to inorganic phosphorus are studied, differences in the results of the two series of experiments are at once apparent. Although the injection of phosphate causes a fall in the serum-calcium the converse is not observed, since when a calcium salt is injected, the serum-inorganic phosphorus is virtually unaffected. The inverse relationship between the serum-calcium and inorganic phosphorus as observed in the dietary experiments therefore appears to operate in one direction only. The direct relationship between magnesium and inorganic phosphorus observed in the dietary experiments receives no support from the results of the injections, since while the

injection of phosphate leaves the serum-magnesium unaffected, the injection of magnesium salts depresses the inorganic phosphorus so that the two constituents are actually in inverse relationship.

The lack of reciprocity between calcium and inorganic phosphorus on the one hand and between inorganic phosphorus and magnesium on the other which is indicated by the results of the injection experiments appears to find an explanation when the relative concentrations of these constituents in the blood are considered. Normally the calcium of serum is more than equivalent to the inorganic phosphorus, so that it might be anticipated that, when more phosphate was introduced into the blood-stream, combination with calcium would occur with the deposition in some suitable site of calcium phosphate and a consequent fall in the serum-calcium value. Since calcium is present in excess of inorganic phosphorus, it is hardly surprising that the introduction of further calcium produces no fall in the inorganic phosphorus. Similarly, the inorganic phosphorus of serum is more than equivalent to the magnesium, so that, when more magnesium is introduced, it seems reasonable to expect that some of the magnesium will combine with inorganic phosphorus, the disappearance of which from the blood will lead to a fall in the serum-inorganic phosphorus. On the other hand, the addition of further phosphate when inorganic phosphorus is already in excess need hardly be expected to cause a fall in the serum-magnesium.

The injection experiments suggest that fluctuation of the serum-inorganic phosphorus is the chief controlling factor in the calcium-inorganic phosphorus relationship observed in the dietary experiments. This is in harmony with the explanation suggested by Fraser [1932] who regards the relationship as dependent on the periodical liberation of calcium phosphate from the bones. The calcium plays a passive rôle, in that calcium deficiency causes calcium phosphate to leave the depots with consequent rise in the serum-inorganic phosphorus value, whereas the presence of a sufficiency of calcium in the blood leads to a discontinuance of the process, so that the serum-inorganic phosphorus tends to fall. It is obvious that the depots must normally be replenished from time to time and it may fairly be assumed that a rise in the serum-inorganic phosphorus is instrumental in this effect leading to a deposition of calcium phosphate in the bones. Regarded from this aspect, the mechanism of the reciprocal relationship might be stated thus: A rise in the serum-inorganic phosphorus leads to a fall in the serum-calcium by deposition of calcium phosphate in bone, while a fall in the phosphorus value, as by excess of excretion over intake, allows liberation of calcium phosphate from the bones to proceed with consequent rise in the serum-calcium value.

Summary.

1. With a view to confirming earlier observations of the effects of the inorganic constituents of diet on the relationships between the concentrations of calcium, magnesium and inorganic phosphorus in the serum of the rabbit, solutions containing the appropriate ions have been injected subcutaneously and their effects on the three constituents compared with those noted previously.

2. The inverse relationship existing between the concentrations of calcium and magnesium in the serum, first observed when rabbits are kept on certain diets, has been confirmed by the subcutaneous injection of calcium and magnesium salts.

3. The inverse relationship between the serum-calcium and inorganic phosphorus and the direct relationship between the serum-magnesium and inorganic phosphorus is only supported in so far as the injection of disodium phosphate

causes a fall in the serum-calcium. The injection of the gluconate, laevulate and lactate of calcium has no constant effect on the serum-inorganic phosphorus, nor does the injection of phosphate affect the serum-magnesium. The injection of the sulphate and lactate of magnesium depresses the serum-inorganic phosphorus, and the relationship is therefore the converse of that observed in the dietary experiments.

4. The above findings are discussed in relation to the concentration of calcium, magnesium and inorganic phosphorus, normally existing in the blood.

5. The injection of sodium sulphate depresses the serum-calcium and inorganic phosphorus but leaves the serum-magnesium unchanged.

6. The injection of sodium chloride has no significant effect on the serum-calcium, magnesium or inorganic phosphorus.

7. Small haemorrhages cause a slight temporary depression of the serum-calcium.

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REFERENCES.

Apitzsch (1931). *Z. ges. exp. Med.* **76**, 313.
Binger (1917). *J. Pharm. Exp. Ther.* **10**, 105.
Bomskov (1930). *Arch. exp. Path. Pharm.* **157**, 220.
Bourne and Campbell (1932). *Biochem. J.* **26**, 183.
Boyd, Hines and Stearns (1930). *Proc. Soc. Exp. Biol. Med.* **27**, 266.
Brookfield (1933). *Biochem. J.* **27**, 173.
Clark (1920). *J. Biol. Chem.* **43**, 89.
Collip (1926). *Amer. J. Physiol.* **76**, 472.
Condorelli (1926). *Arch. Farm. Sper.* **42**, 193.
Culhane (1927). *Biochem. J.* **21**, 1015.
Dupré and Semeonoff (1931). *J. Biol. Chem.* **94**, 341.
Fraser (1932). *Biochem. J.* **26**, 2166.
Greenwald and Gross (1925). *J. Biol. Chem.* **66**, 201.
Greville (1931). *Biochem. J.* **25**, 1931.
Mendel and Benedict (1909). *Amer. J. Physiol.* **25**, 1, 23.
Meneghetti (1927). *Biochem. Ter. Sper.* **14**, 116.
Moritz (1925). *J. Biol. Chem.* **66**, 343.
Pribl (1929). *Compt. Rend. Soc. Biol.* **102**, 258.
Reding and Slosse (1923). *Compt. Rend. Soc. Biol.* **88**, 644.
Salvesen, Hastings and McIntosh (1924, 1). *J. Biol. Chem.* **60**, 311.
—— —— (1924, 2). *J. Biol. Chem.* **60**, 327.
Schiff (1920). *Jahrb. Kinderheilk.* **91**, 43.
—— and Stransky (1920). *Jahrb. Kinderheilk.* **93**, 205.
Stewart and Percival (1927). *Biochem. J.* **21**, 301.
Stransky (1915). *Arch. exp. Path. Pharm.* **78**, 122.
Tisdall (1922). *J. Biol. Chem.* **54**, 35.

CI. DIRECT FERMENTATION OF MALTOSE[1].

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WILLSTÄTTER designated as "direct fermentation" of disaccharides the fermentation of sugars like sucrose, maltose or lactose without preceding hydrolysis into monosaccharides. The assumption of such direct fermentations conflicts with the prevailing hypothesis that any glycolysis of polysaccharides must be preceded by cleavage of the glucosidic linkages. This classical hypothesis, perhaps first formulated by Emil Fischer and Lintner, has been generally accepted, as it seems to be supported by ample experimental evidence, such as the correlation of fermentability and non-fermentability of carbohydrates by yeasts and other micro-organisms with the presence or absence of specific carbohydrases.

The validity of this theory was questioned by Willstätter and Steibelt [1921]. They found that brewer's yeast generally contained ample maltase to accomplish the conversion of maltose in the wort into glucose for subsequent fermentation. Table I gives a synopsis of the time required for half fermentation and for half hydrolysis of maltose under comparable standard conditions for various yeasts studied in this laboratory and including some examples given by Willstätter and Steibelt [1921] and Willstätter and Bamann [1926, 1]. Munich brewer's yeast (Löwenbräu) ferments maltose rapidly, but can hydrolyse it still faster. With various distiller's yeasts, the time of half hydrolysis is of the same order of magnitude as that of half fermentation, but the maltase content seems to be less than sufficient. A third group, comprising yeast M of the Berlin Institut für Gärungsgewerbe, Vienna distiller's yeast, and New York Lager yeast, is distinguished by a paradoxical lack of maltase. The amount of maltase found is negligible. Although the determination of maltase in such small amounts is not very accurate, even the lowest values for half hydrolysis (highest maltase values) are many times larger than the time required for half fermentation of the same quantity of maltose. The maltase content is far from sufficient to supply the zymase with those amounts of glucose necessary, according to the theory, for the maintenance of the observed swift development of carbon dioxide and alcohol. Willstätter and Oppenheimer [1921–22] observed an analogous discrepancy between the fermentation of lactose and the low amount of lactase in a strain of *Saccharomyces fragilis* Jørgensen.

Before discussing various attempts to reconcile these facts with the classical theory, one might define "direct fermentation" in the light of the present knowledge regarding the zymase complex. The absence of disaccharidase, *e.g.* maltase, as shown by the absence of glucose from the fermenting mixture, leads to the assumption that the sequence of intermediaries in disaccharide fermentation converges at a later stage with that of common hexose fermentation. Thus, we may suspect the existence of disaccharide-phosphates and possibly the transi-

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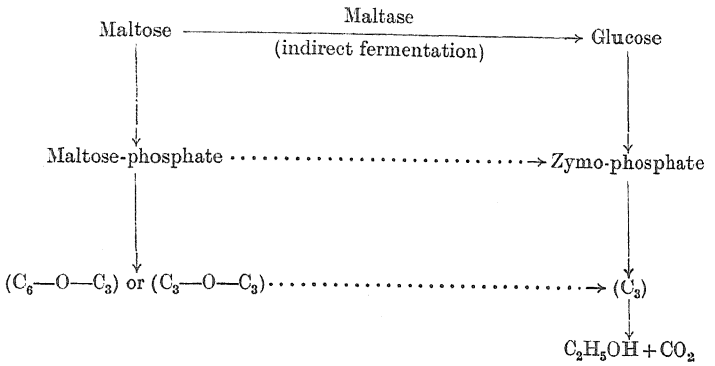
tory formation of $C_6—O—C_3$ or $C_3—O—C_3$ derivatives. The eventual cleavage of the glucosidic linkage in any such molecule could hardly be considered as "maltase" action. The further course of direct fermentation is indicated by the dotted arrows in the diagram.



In order to preserve the classical conception, it may be argued, (1) that the competitive inhibition by glucose, the product of hydrolysis, impairs maltase action in the maltase determination, in contrast to conditions during fermentation when the product of hydrolysis is constantly removed; (2) that the hydrolytic enzyme is regenerated during fermentation; (3) that in these paradoxical cases the method of maltase determination fails to give the full value of maltase activity. The maltase might be present, but not in an extractable form; the active group of the maltase might even be linked with the same colloidal carrier as that component of the zymase system which initiates glucose fermentation, and the glucose molecule would be handed over by a conveyor-like mechanism from the maltase to the zymase without the possibility of glucose being detected or isolated. While such a mechanism would still fall under the definition of direct fermentation, positive proof for it could only be adduced again by the separation of such a "malto-zymase" into maltase and zymase.

Point (1) can easily be refuted since the affinity of glucose for maltase is quantitatively known; thus, the inhibitory effect of the monosaccharide on the enzyme can be evaluated and shown to be of no consequence in comparison with the observed divergencies. Point (2) was raised by Euler and Brunius [1926], when Willstätter and Lowry [1925], and Willstätter and Bamann [1926, 1] succeeded in demonstrating direct fermentation of sucrose and of maltose with yeasts depleted of the respective disaccharidases by treatment with mineral acid or alkali hydroxide. Sucrase determination after fermentation sometimes showed increments, but not sufficient to account for the rate of fermentation. In some instances, *e.g.* in the case of a lactose-fermenting yeast, the enzyme content after fermentation was just as low as before fermentation. No galactosazone, but only lactosazone, could be isolated from a partially fermented lactose solution. A further contribution by Willstätter and Bamann [1926, 2] showed that even yeasts rich in maltase will ferment without antecedent hydrolysis at p_H 5, at which maltase is practically inactive whilst fermentation proceeds at an optimum rate.

The authors feel that this problem forms a part of the general question, whether the metabolism of high-molecular compounds follows exclusively those patterns presented by the action of non-specific laboratory reagents such as acids and alkalis. The following report includes unpublished observations on yeast M made by one of us in 1921 under the direction of Prof. R. Willstätter [Sobotka,

1922], and a series of experiments with a peculiar yeast which was very kindly supplied by Mr Leo Wallerstein.

Yeast M of the Berliner Institut für Gärungsgewerbe, recommended for the fermentation of molasses, is, according to German authors, a mixture of five strains of yeast, among them yeasts II and XII. This statement is hardly in agreement with the fact that yeasts II and XII have maltase values of 1·3 and 4–6, while no sample of yeast M ever contained more than 0·4 maltase units per g. Samples of yeast M of maltase values 0·1–0·05 fermented maltose almost as fast as glucose, except, under the chosen conditions, for a peculiar period of induction of about two hours. Such prolonged inductions have never been observed with live yeast, whilst they are familiar with dry yeast and yeast juice fermentations [Sobotka, 1924]. Contact of the yeast with maltose solution abolishes induction, but even a prolonged stay of the yeast in a maltose medium and frequent renewal of maltose failed to enhance the maltase content of the yeast, while its fermenting power for maltose reached that for glucose during this maltose treatment.

The possibility of maltase formation during maltose fermentation was excluded in the following experiment. A maceration juice prepared according to Lebedev [1912] from an air-dried brewer's yeast, rich in maltase, contained exactly the same amount of maltase as the fresh yeast from which it was derived. A maceration juice from dry yeast M corresponded in its poor maltase content with fresh yeast M, whilst it fermented maltose. This observation supports the assumption of direct fermentation, as it excludes regeneration of maltase during fermentation, which is inconceivable in the cell-free juice. It also corroborates the soundness of the maltase determination with ethyl acetate on fresh yeast, as any cell-bound enzyme, which might have escaped that determination, should have been liberated during maceration.

Proof that the "malto-zymase" is not only free from a maltase component but actually follows a different initial path from "gluco-zymase" was obtained with the yeast from the Wallerstein Laboratories. This strain is distinguished by its ability to live, to ferment and to propagate at the rather acid p_H 2·5. This yeast is free from maltase both at p_H 2·5 and at p_H 6·5, the optimum for the action of yeast-maltase. None the less, it speedily ferments maltose or malt extract media, and, in several experiments, the rate of maltose fermentation exceeded that of glucose fermentation. These observations can be interpreted only on the assumption of direct fermentation of maltose.

Experimental.

Determination of maltase value and maltase units. Willstätter *et al.* [1924] defined as Maltase Unit (M.U.) that amount of enzyme which hydrolyses 2·5 g. maltose hydrate in 50 ml. of a phosphate buffer solution of p_H 6·8 at 30° to the extent of 50 % within one minute. A yeast has the Maltase Value (M.V.) = 1 if it contains 1 M.U. in 1 kg. dry substance.

The M.V. is usually estimated in the following manner. After the dry weight of the yeast has been determined, an amount equivalent to 2·5 g. dry yeast, usually about 10 g. fresh yeast, is liquefied with 1 ml. ethyl acetate, following the procedure of Willstätter and Steibelt [1920]. After cautious neutralisation with a suitable amount of $N/10$ ammonia, the solution is made up to 50 ml.; 40 ml. of this are added to 50 ml. of a 10 % maltose hydrate solution *plus* 5 ml. of $M/3$ phosphate buffer (equal parts of $M/3$ KH_2PO_4 and Na_2HPO_4) and made up to 100 ml. Samples of 25 ml. are withdrawn at appropriate intervals, *e.g.* 10, 20 and 40 minutes, and the action of the enzyme is interrupted by running the sample

into 5 ml. of a $2N$ Na_2CO_3 solution, which at the same time catalyses complete mutarotation of the products of hydrolysis [Hudson, 1908]. The samples are clarified by filtration, if necessary with addition of charcoal. The rotation in a 2 dm. tube of the clarified solution will be found to be $+10{\cdot}80°$ for a sample taken immediately after addition of the enzyme, and $+4{\cdot}40°$ after complete hydrolysis. Thus, percentage hydrolysis can be determined and the time for 50 % hydrolysis can be interpolated by means of the typical reaction curves [see Bamann, 1929].

If 50 % hydrolysis is accomplished, *e.g.* in 70 minutes, 50 ml. of the reaction mixture, equivalent to 1 g. dry yeast, contain 1/70 M.U. Thus, the M.V. of this yeast would be $1000/70 = 14{\cdot}3$.

Fermentation of sugars can be measured in a similar way. 20 ml. of a 5 % sugar solution, saturated with carbon dioxide, are fermented with fresh yeast equivalent to 0·2 g. dry yeast, and the carbon dioxide developed is measured, preferably over mercury. The amount of carbon dioxide developed under 760 mm. Hg pressure and 30° is 282 ml. The time within which half this amount is liberated can be correlated with the time of half hydrolysis under the conditions of the maltase determination, which are all identical except that the double amount of yeast is employed. The values for half fermentation of maltose, given in Table I, were obtained by this procedure.

Table I. *Fermentation and hydrolysis of maltose by various yeasts.*

Yeast	Time of half fermentation mins.	Time of half hydrolysis mins.	Maltase value
Munich brewer's	80	60	33·3
Danish distiller's	255	320	6·2
XII, distiller's	205	300–760	6·6–2·6
New York baker's	600	400–900	5·0–2·2
New York ale	—	900	2·2
Rhine wine	—	500–640	4·0–3·1
New York lager	460	4,000	0·5
Munich brewer's, acid-treated	170	4,000	0·5
Frankenthal distiller's	240	50,000	0·04
Vienna-Stadlau distiller's	1200	8,000–25,000	0·25–0·08
M, distiller's	480	5,200–16,000	0·38–0·12
A, grown at p_H 2·5	800	40,000	0·05

Induction period. Yeasts poor or lacking in maltase ferment maltose, as a rule, only after a delay of 10 minutes to 3 hours under the above conditions. After this induction period which lasts from 2 to 3 hours with yeast M, the development of carbon dioxide soon reaches the linear course typical for alcoholic fermentation. If this period of induction is subtracted from the time of half

Table II. *Fermentation of glucose and maltose by yeast M.*

	Time for half fermentation			Corrected time for half fermentation mins.
	Glucose mins.	Maltose mins.	Induction mins.	
Original sample (1. vii. 21)	200	380	120	260
After glucose passage	270	390	110	280
After 1 maltose passage	250	280	none	—
After 2 maltose passages	240	255	none	—
Same, 5 times washed	205	335	none	—
Original sample (22. vii. 21)	—	390	85	305
After maltose passage	210	220	none	—

fermentation in the case of yeast M, it is found that the maltose fermentation, once it has started, proceeds at the same rate as glucose fermentation. If fresh yeast M is carried through one or two passages in diluted malt extract, the induction period disappears. Maltase determinations in yeast M, after maltose treatment had eliminated the induction period, still yielded values of 9000 and 17,000 minutes as time for half hydrolysis. Analysis of reaction mixtures at the end of the induction period proved the absence of glucose.

Maltase determination in maceration extract. Maceration extract [Lebedev, 1912] was prepared in the following manner. Yeast was dried in a 2 mm. layer between 30° and 40°. One part of dry yeast was incubated at 30° for 4 hours with three parts of water. Three ml. of clear juice, isolated by centrifuging, corresponded to 1 g. dry yeast. Three or six ml. of juice were used in a total volume of 100 ml. buffered solution containing 5 % maltose; fermentation was effectively prevented by the addition of 5 ml. ethyl acetate. The figures given in Table III show that fresh maceration extract from brewer's yeast contains an

Table III. *Maltase determinations in fresh yeasts and maceration extracts.*

	$p_H = 6{\cdot}8$, $t = 30°$				
	Dry yeast equivalent g./100 ml.	Time min.	Decrease in rotation ($l = 2$ dm.)	% hydrolysis	Time of half hydrolysis, calc. for 1 g. dry yeast in 100 ml. min.
Löwenbräu yeast	2·0	40	2·80°	43·8	56
	—	80	3·72	58·2	56
Löwenbräu, maceration extract, 10 ml.	3·33	40	4·14	64·7	63
Same, after 24 hours, 6 ml.	2·0	30	1·74	27·2	235
	—	65	2·52	39·4	235
	—	120	3·25	50·8	330
Yeast M	2·0	160	0·25	3·9	7000
Same	2·0	450	1·00	15·6	5200
Yeast M, maceration extract, 6 ml.	2·0	1200	1·92	30·0	7800
Same, after 24 hours, 6 ml.	2·0	91	0·10	1·6	14,000
	—	132	0·18	2·9	13,500

amount of maltase identical with that found in the fresh yeast. It deteriorates like other enzymes in maceration extract rather rapidly. On the other hand, a fresh extract made from dried yeast M contained minute amounts of maltase only, of the same order of magnitude as the fresh yeast.

Yeast A. The yeast obtained from Mr Wallerstein, briefly designated yeast A, grows in a 10 % solution of malt extract the p_H of which is brought to 2·5–2·8 by addition of 0·8 volume % concentrated sulphuric acid. No maltase was found in this yeast when it was tested either at p_H 6·5 or at p_H 2·5. However, it fermented both maltose and glucose.

Fermentation was carried out in a Barcroft-Warburg apparatus, both in oxygen and in nitrogen, and the rate of carbon dioxide development was measured. This varied between 100 and 250 mm.^{3}/30 minutes, when an amount of fresh yeast equivalent to 4 mg. dry yeast was allowed to act on 50 mg. sugar in a total volume of 2 ml. $M/20$ primary phosphate solution. A comparison between maltose and glucose fermentation showed no significant difference between the two sugars. However, with one batch, the fermentation of maltose

was definitely faster than that of glucose. 208 mm.^{3} carbon dioxide were developed from maltose in thirty minutes as compared with 161 mm.^{3} from glucose under anaerobic conditions; under aerobic conditions the figures were 129 mm.^{3} for maltose and 73 mm.^{3} for glucose.

REFERENCES.

Bamann (1929). In Oppenheimer, Die Fermente und ihre Wirkungen, vol. III, p. 857. (Leipzig.)
Euler and Brunius (1926). *Z. physiol. Chem.* **160**, 242.
Hudson (1908). *J. Amer. Chem. Soc.* **30**, 1160.
Lebedev (1912). *Ann. Inst. Pasteur* **26**, 8.
Sobotka (1922). Thesis, p. 39, Munich.
—— (1924). *Z. physiol. Chem.* **134**, 1.
Willstätter and Bamann (1926, 1). *Z. physiol. Chem.* **151**, 242.
—— —— (1926, 2). *Z. physiol. Chem.* **152**, 202.
—— Kuhn and Sobotka (1924). *Z. physiol. Chem.* **134**, 224.
—— and Lowry (1925). *Z. physiol. Chem.* **150**, 287.
—— and Oppenheimer (1921–22). *Z. physiol. Chem.* **118**, 168.
—— and Steibelt (1920). *Z. physiol. Chem.* **111**, 157.
—— —— (1921). *Z. physiol. Chem.* **115**, 211.

CII. THE RELATION OF INTERMEDIARY METABOLIC PRODUCTS TO ARGINASE ACTIVATION.

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The catheptic enzymes, which are concerned with proteolysis in animal tissue, undergo activation when treated with certain substances, such as HCN or H_2S. These characteristic activation phenomena became of increased physiological interest with the discovery of the occurrence in tissues of natural activators, as for example, zookinase [Waldschmidt-Leitz *et al.*, 1930], which has been shown by Waldschmidt-Leitz and Purr [1931] to be identical with reduced glutathione. The same activator has been described by Grassmann *et al.* [1931] for the plant proteases (papain, bromelin and yeast-proteinase). In later experiments it was established [Waldschmidt-Leitz, Scharikova and Schäffner, 1933] that arginase, another enzyme concerned with intermediary protein metabolism, is also activated directly by metal complexes of SH compounds, for example, by reduced glutathione and ferrous or cuprous ions.

The interpretation of these activations as a specific function of the sulphydryl group [Waldschmidt-Leitz, Weil and Purr, 1933] cannot be considered as valid, for we have found that other types of substances, as for example, ascorbic acid-$Fe^{=}$, methylglyoxal-$Fe^{=}$, and alloxan-$Fe^{=}$ exercise the same activating effect on arginase as is obtained with the sulphydryl-$Fe^{=}$ system (see Tables V, II and I). These newly demonstrated activators however possess in common the property of forming reversible redox systems. The hypothesis of a connection between arginase activation and the oxidation-reduction potential, for the establishment of which oxygen [Waldschmidt-Leitz, McDonald *et al.*, 1933] or hydrogen [Edlbacher *et al.*, 1933] is necessary, is in harmony with the experimental findings and accounts for the activations observed with the above-mentioned compounds.

Investigations reported by various writers during the past year have shown that in general these oxidations and reductions are regulated by reversible redox systems, which in the presence of available oxygen or hydrogen show variable potentials. Of significance to the investigation is the fact that the potential is determined not by the total amount of the system present but only by the ratio of the reduced to the oxidised phase. The total amount acts to a certain extent however as a buffer. With a constant ratio of reduced form to oxidised form, it is apparent that the redox potentials will vary also with the p_H. With increasing alkalinity they become more negative (more intense), and with increasing acidity, less negative (less intense). The particular physiological significance of such biological oxidations and reductions is dependent on the one hand on the assumption of a capacity to absorb such reduction potential (specificity) and on the other hand on the intensity of such potential. It will

thus be necessary, in enzyme activations, to distinguish between a specificity factor, and an intensity (activation) factor, which latter is known to be influenced by p_H and is responsible for the attainment of the full activity of enzymes.

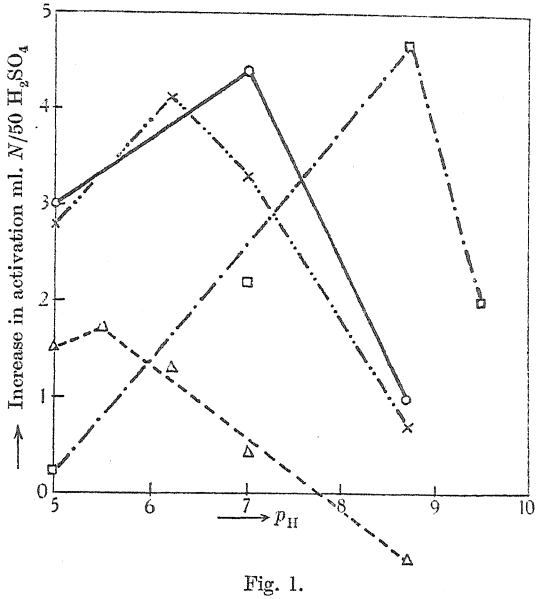

Fig. 1.

Cysteine-iron ($Fe^{=}$). — · — Cysteine.
- - - - Bivalent iron. —··— Ascorbic acid-iron ($Fe^{=}$).

From the curves (Fig. 1) which represent the experiments with $Fe^{=}$, cysteine, cysteine-$Fe^{=}$ and ascorbic acid-$Fe^{=}$, it is clear that the optimum development of the intensity factors of arginase, which is the development of the full activity, is dependent, as previously mentioned, on the p_H. Thus one obtains, by shifting the p_H to the alkaline side, the same activation intensity optimum as is obtained at neutral reaction by the addition of bivalent iron. While cysteine in acid medium is indifferent or even inhibiting, bivalent iron shows, to a less extent, the opposite picture. Bivalent iron therefore acts in a certain sense as a p_H buffer with respect to the development of the specific intensity potential which is required by the arginase, and it may also act in the same way in the organism. With ascorbic acid-$Fe^{=}$, the activation intensity optimum is shifted still more to the acid side than is the case with cysteine-$Fe^{=}$. In agreement with these results, Hill and Michaelis [1933] have found that the potential developed in the system alloxantin-$Fe^{=}$ is also dependent on the p_H, the bivalent iron apparently being a determining factor.

In a previous communication [Purr, 1933] an activation of arginase by ascorbic acid-$Fe^{=}$ was reported. In connection with this finding, the question was raised as to what extent other intermediary metabolic products exert a regulating effect on intracellular enzymic syntheses or hydrolyses. The experiments here reported, which have been carried through from this point of view, have shown that the activation mechanism of this enzyme is probably connected with a specific oxidation-reduction potential, and is not due simply to a specific effect of the SH group.

Experimental.

The activation of arginase.

The organs studied were rat liver and transplantable rat sarcoma, Philadelphia # 1[1]. Immediately after extirpation these tissues were cut up with scissors and frozen by dipping into liquid nitrogen, then pulverised by crushing with a hammer. This finely crushed material was suspended as follows: cancer (Philadelphia # 1 rat sarcoma) in 90 % glycerol (1: 10); rat liver in 90 % glycerol (1: 10); glycerol extract from liver acetone-ether dry preparation

Table I. *The effect of alloxan, allantoin, alloxantin and uric acid.*

The enzyme was activated by 0·5 ml. 0·1 *N* $FeSO_4$ or 20·0 mg. alloxan, 20·0 mg. allantoin, 20·0 mg. alloxantin, 20·0 mg. uric acid, respectively. p_H 7.

	Enzyme		
Activator system	Liver acetone-ether dry preparation (ml. 0·02 *N* H_2SO_4)	Rat liver suspension (ml. 0·02 *N* H_2SO_4)	Cancer suspension (Philadelphia # 1 rat sarcoma) (ml. 0·02 *N* H_2SO_4)
Initial activity	6·8	7·0	2·3
$Fe^{=}$	14·6	6·4	3·3
Cysteine	7·1	8·1	5·8
Cysteine-$Fe^{=}$	20·4	15·2	7·9
Alloxan	8·4	—	3·2
Alloxan-$Fe^{=}$	19·3	—	8·0
Allantoin	8·6	—	—
Allantoin-$Fe^{=}$	15·4	—	—
Alloxantin	—	7·9	—
Alloxantin-$Fe^{=}$	—	9·6	—
Uric acid	6·2	—	—
Uric acid-$Fe^{=}$	11·6	—	—

Table II. *The effect of methylglyoxal, pyruvic acid and* d-*lactic acid.*

The enzyme was activated by 0·5 ml. 0·1 *N* $FeSO_4$ or 16·0 mg. methylglyoxal, 20·0 mg. pyruvic acid, 20·0 mg. *d*-lactic acid, respectively. p_H 7.

	Enzyme		
		Cancer suspension (Philadelphia # 1 rat sarcoma) (ml. 0·02 *N* H_2SO_4)	
Activator system	Rat liver suspension (ml. 0·02 *N* H_2SO_4)	No. 1	No. 2
Initial activity	7·0	7·7	3·4
$Fe^{=}$	6·4	7·6	4·7
Cysteine	8·1	10·8	4·3
Cysteine-$Fe^{=}$	15·2	10·8	8·2
Methylglyoxal	—	2·0	4·0
Methylglyoxal-$Fe^{=}$	—	18·6	7·7
Pyruvic acid	7·1	—	—
Pyruvic acid-$Fe^{=}$	6·3	—	—
d-Lactic acid	6·2	—	—
d-Lactic acid-$Fe^{=}$	6·7	—	—

[1] The pathological description of this transplantable rat sarcoma, Philadelphia # 1, can be found in the article "Ueber die Enzyme in Tumoren," by Waldschmidt-Leitz, McDonald *et al.* [1933].

(1 : 10). The amounts used were: 5·0 ml. cancer suspension; 0·25 ml. liver suspension; 0·5 ml. extract of acetone-ether dry preparation. For activation 2·0 ml. neutral cysteine-HCl solution (containing 20 mg. cysteine-HCl) or 0·5 ml. 0·1 *N* $FeSO_4$, respectively, were added to the enzyme and allowed to stand 1 hour at p_H 7 at 30°. 10 ml. of 1 % arginine carbonate and 5·0 ml. 0·1 *M* glycine buffer of p_H 9·5 were then added and the reaction mixtures allowed to stand 60 minutes at 30°.

Results are shown in ml. 0·02 *N* H_2SO_4, as is customary.

Table III. *The effect of thiolacetic acid.*

The enzyme was activated by 0·5 ml. 0·1 *N* $FeSO_4$ or 20·0 mg. thiolacetic acid, respectively. p_H 7.

Activator system	Enzyme. Cancer suspension (Philadelphia # 1 rat sarcoma) (ml. 0·02 *N* H_2SO_4)
Initial activity	6·0
$Fe^{=}$	10·5
Cysteine	8·1
Cysteine-$Fe^{=}$	14·5
Thiolacetic acid	6·8
Thiolacetic acid-$Fe^{=}$	12·5

Table IV. *The effect of bivalent iron in complex combination.*

(Potassium ferrocyanide, $Fe^{=}$-dimethylglyoxime and αα′-dipyridyl[1].)

The enzyme was activated by 20·0 mg. potassium ferrocyanide; 20·0 mg. dimethylglyoxime; 5 mg. dipyridyl; (A) after 1 hour's standing with dipyridyl, $FeSO_4$ was added and the mixture allowed to stand another hour, p_H 7; (B) the same with cysteine instead of $FeSO_4$; (C) the same with cysteine-$Fe^{=}$; (D) after 1 hour's standing with dipyridyl, and another after addition of $FeSO_4$, cysteine-$Fe^{=}$ was added and the mixture allowed to stand an additional hour.

Activator system	Enzyme: Liver acetone-ether dry preparation (ml. 0·02 *N* H_2SO_4)	Enzyme: Rat liver suspension (ml. 0·02 *N* H_2SO_4)
Initial activity	6·8	7·0
$Fe^{=}$	14·6	6·4
Cysteine	7·1	8·1
Cysteine-$Fe^{=}$	20·4	15·2
Potassium ferrocyanide	3·6	—
Dimethylglyoxime	3·3	—
Dimethylglyoxime-$Fe^{=}$	8·3	—
αα′-Dipyridyl	—	6·2
(A)	—	1·0
(B)	—	5·0
(C)	—	14·4
(D)	—	3·0

From these figures it is evident that ferrous compounds in their rôle as activator for arginase are not interchangeable with bivalent iron in complex combination. It is also shown that ferrodipyridyl (A) inhibits irreversibly the action of arginase.

[1] The authors wish to thank Dr L. Michaelis, of the Rockefeller Foundation for Medical Research, for his kindness in supplying the αα′-dipyridyl.

Table V. *The effect of* p_H *(specificity of activation).*

The enzyme was activated by 0·5 ml. 0·1 *N* $FeSO_4$ or by 20 mg. cysteine-HCl or 16 mg. ascorbic acid ("chinoin") respectively, and allowed to stand 1 hour at p_H 5·0, 5·5, 6·2, 7·0, 8·7, 9·5 at 30° (the p_H was controlled). The activation of arginase was determined as usual at p_H 9·5. (Enzyme influence in ml. 0·02 *N* H_2SO_4.)

	Enzyme. Rat liver suspension					
p_H	5·0	5·5	6·2	7·0	8·7	9·5
Activator system						
Initial activity	—	—	—	4·7	—	—
$Fe^{=}$	6·2	6·4	6·0	5·1	4·0	—
Cysteine	4·5	—	—	6·9	9·4	6·7
Cysteine-$Fe^{=}$	7·7	—	—	9·1	5·7	—
Ascorbic acid-$Fe^{=}$	7·5	—	8·8	8·0	5·4	—
Increase in activation						
$Fe^{=}$	1·5	1·7	1·3	0·4	−0·7	—
Cysteine	0·2	—	—	2·2	4·7	2·0
Cysteine-$Fe^{=}$	3·0	—	—	4·4	1·0	—
Ascorbic acid-$Fe^{=}$	2·8	—	4·1	3·3	0·7	—

SUMMARY.

It has been shown that various intermediate products of metabolism, in combination with ferrous iron, are able to activate arginase. The activation mechanism of this enzyme is probably connected with a specific oxidation-reduction potential and is not due simply to a specific effect of the SH group.

We wish to express our thanks to Doctor Ellice McDonald, Director, for his interest and support throughout this work.

REFERENCES.

Edlbacher, Kraus and Leuthardt (1933). *Z. physiol. Chem.* **217**, 89.
Grassmann, Schoenebeck and Eibeler (1931). *Z. physiol. Chem.* **194**, 124.
Hill and Michaelis (1933). *Science*, **78**, 485.
Purr (1933). *Biochem. J.* **27**, 1703.
Waldschmidt-Leitz, Schäffner, Bek and Blum (1930). *Z. physiol. Chem.* **188**, 29.
—— McDonald und Mitarbeiter (1933). *Z. physiol. Chem.* **219**, 120.
—— and Purr (1931). *Z. physiol. Chem.* **198**, 260.
—— Scharikova and Schäffner (1933). *Z. physiol. Chem.* **214**, 75.
—— Weil and Purr (1933). *Z. physiol. Chem.* **215**, 64.

CIII. PYRUVIC ACID IN THE DISSIMILATION OF GLUCOSE BY THE PROPIONIC ACID BACTERIA[1].

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(*Received February 22nd, 1934.*)

ALTHOUGH Van Niel [1928] postulated that pyruvic acid is formed during the dissimilation of glucose by the propionic acid bacteria, the keto-acid has never been identified as an intermediate product. Van Niel assumed that pyruvic acid hydrate was formed and subsequently dehydrogenated to yield acetic acid, carbon dioxide and two atoms of hydrogen, the latter serving to reduce suitable hydrogen acceptors such as methylglyoxal to propionic acid.

$$CH_3.C(OH)_2.COOH \rightarrow CH_3COOH + CO_2 + 2H \qquad .....(1).$$

This assumption was necessitated by the failure to demonstrate the conversion of acetaldehyde into acetic acid or the presence of acetaldehyde itself during the fermentation of glucose by the propionic acid bacteria, although acetaldehyde would be a normal product of the decarboxylation of pyruvic acid.

By assuming a dehydrogenation of pyruvic acid hydrate, the absence of acetaldehyde is accounted for.

Van Niel based his conclusion of the presence of pyruvic acid largely upon its ease of fermentation by the propionic acid bacteria with formation of propionic acid, acetic acid and CO_2 in substantially theoretically correct proportions.

Actual proof of the intermediate formation of pyruvic acid from glucose by propionic acid bacteria would evidently lend considerable support to Van Niel's theory of dissimilation of glucose by these organisms.

In the present communication we offer such proof in the form of the isolation and identification of pyruvic acid in the fermentation of glucose by *Propionibacterium arabinosum*.

EXPERIMENTAL.

The culture of *Propionibacterium arabinosum* used in this study was kindly furnished by Dr E. R. Hitchner of the University of Maine and was described in detail by Werkman and Brown [1933].

The fermentations were carried out in a medium of Bacto yeast extract 0·5 %, glucose 2·0 %, and calcium carbonate 2 %. Either sodium bisulphite (0·5 %) or calcium sulphite (1·0 %) was included in the medium as a fixative. Constituents of the medium were sterilised separately and mixed. A number of 500 ml. flasks each containing 300 ml. of medium were prepared and the fermentations carried out anaerobically by bubbling oxygen-free nitrogen through the medium. The inoculum was a five-day culture of the organism in glucose yeast extract broth added in quantity equivalent to 5 % by volume of

[1] Supported by Industrial Science Research Funds of Iowa State College.

the medium. Incubation was at 30°. Growth was luxuriant and the glucose was completely utilised by the tenth day of fermentation. The cultures were proven pure by microscopic examination and colony formation in shake tubes and on agar plates.

Both the sodium bisulphite and the calcium sulphite were effective in fixing pyruvic acid. The results were substantially similar with the two fixatives. The following experiment is typical. Tests for acetaldehyde following distillation of the medium according to the method of Peterson and Fred [1920] were negative. Upon addition of 2 : 4-dinitrophenylhydrazine to the medium an abundant precipitate was obtained which was dissolved in sodium carbonate solution and reprecipitated by acidification. After purification of the 2 : 4-dinitrophenylhydrazine precipitate, the melting-point was 212·0°; a mixture with authentic pyruvic acid 2 : 4-dinitrophenylhydrazone (M.P. 213° [*cf.* Allen, 1930]) melted at 212°.

A *p*-nitrophenylhydrazone was likewise prepared from the fermentation solution; it had M.P. 218·5° alone and 218·5° when mixed with authentic pyruvic acid *p*-nitrophenylhydrazone (M.P. 219°).

Discussion.

This identification of pyruvic acid in the fermentation of glucose by *Propionibacterium arabinosum* supports the scheme of glucose dissimilation proposed by Van Niel. The facts that pyruvic acid is fixed during the fermentation and that no acetaldehyde can be detected do not indicate a decarboxylation of pyruvic acid. Carboxylase is reported by Neuberg and Reinfurth [1920] to act upon the bisulphite addition compound of pyruvic acid to form the acetaldehyde bisulphite compound, so that in the presence of carboxylase, fixation of pyruvic acid by sulphite should not prevent simultaneous formation of acetaldehyde. Isolation of pyruvic acid was successful in the present case, apparently owing to the inability of *P. arabinosum* to decarboxylate the pyruvic acid bisulphite compound which is not itself subject to hydration and subsequent dehydrogenation as is pyruvic acid.

The reason for the reported failure of Virtanen [1923] to secure growth of the propionic acid bacteria in the presence of sulphite is not apparent.

The authors found no account in the literature of a similar isolation of pyruvic acid from a sulphite-containing culture of actively proliferating bacteria, although Cook [1930] identified pyruvic acid in a fermentation by resting *Bact. coli* using $NaHSO_3$ as a fixative. This fact may well be the result of the unusual mechanism of the dissimilation of glucose brought about by the propionic acid bacteria in which acetaldehyde does not occur as an intermediate product. This mode of formation of acetic acid and carbon dioxide from pyruvic acid explains their formation in the ratio of 1 : 1, a ratio that the authors have not been able to alter.

With regard to the mode of formation of the pyruvic acid from glucose, the evidence is fragmentary; possibly pyruvic acid arises from the dehydrogenation of methylglyoxal hydrate or lactic acid. The latter substance probably occurs as an intermediate only under certain conditions when it is formed as a stabilisation product. Its occurrence has been frequent in large fermentations conducted in this laboratory.

SUMMARY.

Pyruvic acid has been isolated by fixation with sulphite in the fermentation of glucose by *Propionibacterium arabinosum*.

Acetaldehyde has not been detected in these experiments by the sulphite fixation method.

The scheme proposed by Van Niel for the intermediate breakdown of pyruvic acid is shown to be in agreement with experimental results.

REFERENCES.

Allen (1930). *J. Amer. Chem. Soc.* **52**, 2955.
Cook (1930). *Biochem. J.* **24**, 1526.
Neuberg and Reinfurth (1920). *Ber. deutsch. chem. Ges.* **53** B, 1039.
Peterson and Fred (1920). *J. Biol. Chem.* **44**, 29.
Van Niel (1928). The propionic acid bacteria. Dissertation, Delft.
Virtanen (1923). *Soc. Sci. Fennica, Comment. Physico-Math.* **1**, 36.
Werkman and Brown (1933). *J. Bact.* **26**, 393.

CIV. STUDIES ON THE KINETICS OF HAEMOLYTIC SYSTEMS.

V. A FURTHER INVESTIGATION OF THE INHIBITION PRODUCED BY PLASMA AND SERUM.

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(*Received February 23rd, 1934.*)

If a quantity of plasma or serum is added to a haemolytic system containing a quantity c_1 of a lysin such as saponin or one of the bile salts, the time t_2 required for complete lysis is longer than the time t_1 which would be required if the quantity c_1 were to act in the absence of the added plasma or serum. It seems, indeed, that a quantity of lysin Δ is rendered inactive by the plasma, the remainder of the lysin, c_2, producing lysis in the time $t_2 > t_1$. The problem is to find a simple and satisfactory relation between the variables.

One of us attempted to do this in 1923 [Ponder, 1923], and influenced by the idea that plasma-proteins might "adsorb" some of the lysin, sought a relation between Δ, the amount "adsorbed," and c_2, the amount left free. These two quantities were found to be related by an expression not unlike the adsorption isotherm,

$$\Delta = \alpha c_2^{1/\mu} \qquad \ldots\ldots(1),$$

where α and μ are constant, provided that any one amount of plasma M is used as the inhibitor. Plotting $\log \Delta$ against $\log c_2$ thus resulted in a straight line with a slope of $1/\mu$, but when the value of M was varied, μ was found to vary from about 3·5 when M was 0·04 ml. to about 0·035 when M was 0·00125 ml. The straight lines corresponding to various values of M accordingly cut one another: "an impossible state of affairs" [Ponder, 1925].

This difficulty was in part overcome in 1925 by the observation that the curves obtained by plotting Δ against c_2 do not pass through the origin, but cut the c_2 axis at different levels. To allow for this, expression (1) was re-written

$$\Delta = \alpha (c_2 - K)^{1/\mu} \qquad \ldots\ldots(2),$$

where K is the intercept on the c_2-axis just referred to. The plotting of $\log \Delta$ against $\log (c_2 - K)$ now resulted in a series of parallel straight lines with $\mu = 1{\cdot}75$: this is a much simpler state of affairs, offset, however, by the fact that no good explanation was forthcoming either for the existence of K or for the fact that K was found to vary with M in a quite inexplicable way [Ponder, 1925].

In view of this new difficulty, and in view of the possibility that the premises underlying the analysis might be altogether wrong, a very rigorous test was applied [Ponder, 1932]. If the effect of the added inhibitor is to render lysin inert, and not to interfere with the haemolytic reaction in any other way, we must have the following relations fulfilled,

$$\left.\begin{aligned} \Delta_1/\Delta_2 &= 1 \\ \Delta_1 &= c_1 - 1{\cdot}2c_2 \\ \text{and} \quad \Delta_2 &= c_1 - 1{\cdot}2c_3 \end{aligned}\right\} \qquad \ldots\ldots(3),$$

where c_1 is the initial amount of lysin in the system containing the plasma, c_3 the quantity apparently left free after some of the lysin Δ_2 has been rendered inert as judged by the time required for complete haemolysis, and c_2 the quantity left free after some of the lysin Δ_1 has been rendered inert, as judged by the time required to produce 50 % (or any other percentage) haemolysis. It was found that this condition was very exactly fulfilled, and so the hypothesis that the plasma inactivated lysin without interfering with the other properties of the system was supported by evidence not easy to set aside.

The present situation is therefore very unsatisfactory. The assumption that the plasma reacts with the lysin so as to render some of it inert is apparently sound, but the relation between Δ and c_2 cannot be left as it stands, for either we have to explain the variation of μ with M, or we have to introduce a third constant K, itself a function of M, and devoid of physical meaning. The following investigations will therefore be divided into three parts.

1. Remembering that all previous analyses have been based on data obtained by plotting "short" time-dilution curves, we shall show that essentially the same relations result from analysing data based on the more accurate "long" time-dilution curves [see Ponder and Yeager, 1930].

2. We shall show that the order of the fundamental reaction between cell and lysin is not changed as a result of the presence of the inhibitor, *i.e.* we shall confirm by independent means the result of the test contained in expression (3).

3. Abandoning the idea of an "adsorption," we shall seek a relation between Δ and c_1. This will be found to involve the constants α, μ and K, but the last mentioned will be assigned an obvious physical meaning, and it will be shown that μ is independent of the amount of inhibitor used. In this way the problem will be solved in terms of a single expression, which, although empirical, has a real physical significance. It will further be shown that we are now able to predict, in a qualitative sense at least, the changes which are observed in the amount of inhibition when the number of cells in the systems is varied.

Table I.

	δ_1	c_1	t	δ_2	c_2	Δ
Saponin *plus* 0·02 ml. serum	30,000	66·7	4·0	62,000	32·4	34·3
	35,000	57·1	10·2	75,000	26·6	30·4
	40,000	50·0	30·0	87,000	23·0	27·0
	45,000	44·5	90·0	99,000	20·2	24·3
Saponin *plus* 0·16 ml. serum	30,000	66·7	2·5	54,000	36·7	30·0
	40,000	50·0	11·0	78,000	25·6	24·4
	45,000	44·5	26·0	86,000	23·2	21·3
	50,000	40·0	120·0	101,000	19·8	20·2
Saponin *plus* 0·01 ml. serum	35,000	57·1	3·5	56,000	35·7	21·4
	40,000	50·0	5·1	66,000	30·3	19·7
	45,000	44·5	9·0	76,000	26·3	18·2
	50,000	40·0	16·0	84,000	23·8	16·2
	55,000	36·3	37·0	89,000	22·4	13·9
Saponin *plus* 0·006 ml. serum	40,000	50·0	2·6	55,000	36·3	13·7
	50,000	40·0	6·1	72,000	27·8	12·2
	55,000	36·3	11·0	79,000	25·3	10·7
	60,000	33·3	26·0	86,000	23·2	10·1
	65,000	30·3	48·0	91,000	22·0	8·0
Saponin *plus* 0·004 ml. serum	45,000	44·5	3·5	56,000	35·7	8·8
	50,000	40·0	4·5	63,000	31·8	8·2
	55,000	36·3	7·0	70,000	28·6	7·7
	60,000	33·3	10·5	77,000	26·0	7·3
	65,000	30·3	40·4	89,500	23·7	6·6

Data for "long" time-dilution curves.

These are presented in Table I, and arranged in exactly the same way as in the Table on p. 486 of the paper which contains the results obtained from "short" time-dilution curves [Ponder, 1925]. All the figures refer to time-dilution curves for saponin and a standard suspension of human red cells at 30°. Values of c_1, c_2 and Δ are in γ.

The effect of added plasma on the order of the fundamental reaction.

The time-dilution curves for systems containing various quantities of plasma (or serum) can be individually subjected to analysis for the values of the constants in the equation which describes them,

$$kt=\frac{p}{p-1}\cdot\left\{c^{\frac{p-1}{p}}-(c-x)^{\frac{p-1}{p}}\right\} \qquad \ldots\ldots(4),$$

and, using the data given *in extenso* above together with those for some intermediate curves, we get the values shown in Table II.

Table II.

Serum, ml.	$n\ (=1/p)$	k	x	$R\ (=x_1/x_2)$
0·0	2·6	0·00213	19·0	1·00
0·02	2·6	0·000557	40·0	2·11
0·016	2·6	0·000813	36·4	1·92
0·010	2·6	0·00104	30·8	1·62
0·008	2·6	0·00108	28·6	1·51
0·006	2·6	0·00147	26·7	1·41
0·004	2·6	0·00152	24·1	1·27
0·002	2·6–2·7	0·00174	23·0	1·21

Thus n is constant, irrespective of the quantity of inhibitor added, as required if the test in expression (3) is to apply. When the analysis of the curves is carried out in this way, k decreases as M increases, but this is only because the analysis in terms of expression (4) takes no account of the inhibitor rendering lysin inert. The decreasing value of k is really only another way of expressing the relation of Δ to c_1, as in expression (5).

The accuracy with which the various time-dilution curves are described by expression (4), with the above values of the constants inserted, is really quite surprising.

Results of plotting Δ against c_1.

Plotting Δ against c_2 results in exactly the same type of curve as in previous investigations, the curves corresponding to each value of M cutting the c_2-axis at various levels, and being described with a fair degree of accuracy by expression (2), which contains the arbitrary constant K, a function of M. Plotting Δ against c_1, on the other hand, results in a series of curves which cut the c_1-axis at a single point, so that $\Delta=f(c_1-K)$, K now being a constant for all values of M. (This is clearly shown in Fig. 1, in which the two methods of plotting are compared.) To find a meaning for K, consider the steps of the analysis in detail. The time-dilution curve in the absence of inhibitor has an asymptote at $c_{1\infty}$, for which concentration $t=\infty$. Imagine a time-dilution curve for a system containing an indefinitely small amount of plasma: this curve will differ insensibly from the "standard" curve, and will have an asymptote $c_{2\infty}$, differing insensibly from $c_{1\infty}$. Thus when Δ tends to zero, c_2 will tend to c_1, and the curve obtained by plotting Δ against c_1 will cut the c_1-axis at a point $c_1=c_{1\infty}$, $\Delta=0$, and this will be the intercept K, equal to $c_{1\infty}$. The constant K is therefore equal to the asymptotic

concentration for the "standard" curve with which the time-dilution curve for the system containing plasma is compared, and expression (2) becomes

$$\Delta = \alpha (c_1 - c_{1\infty})^{1/\mu} \qquad \ldots\ldots(5).$$

Plotting $\log \Delta$ against $\log (c_1 - c_{1\infty})$ now gives a series of parallel straight lines for which $\mu = 2{\cdot}0$ (in this case) and for which α has the following values:

Serum, ml.	α
0·02	4·67
0·016	4·27
0·010	3·47
0·006	2·57
0·004	1·74

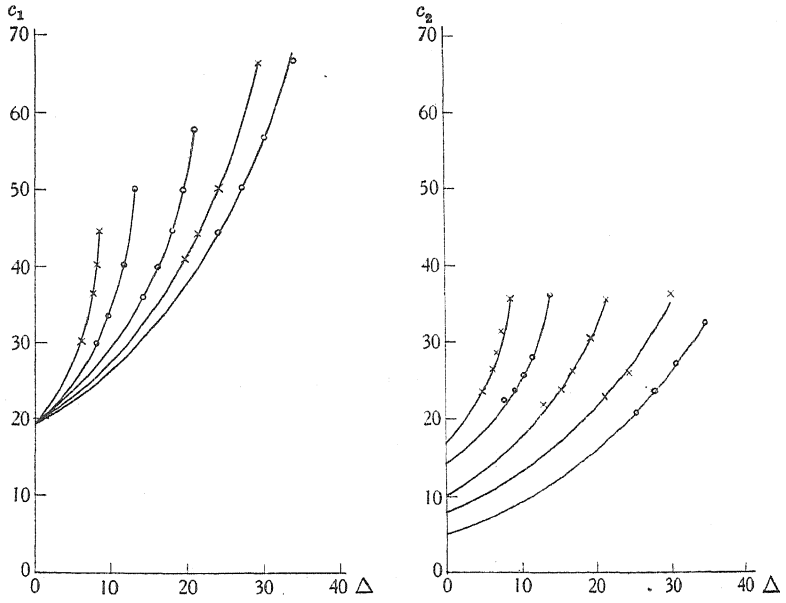

Fig. 1. To show the result of plotting Δ against c_1 and c_2 respectively. The five different curves correspond to five different quantities of serum.

The value of μ is therefore constant for all values of M, while the value of α decreases as M decreases. As has previously been remarked, it does not decrease linearly with M, and small amounts of inhibitor are relatively more effective than larger amounts.

Expression (5) accordingly has a comparatively simple physical meaning, and the only curious thing about it is that the quantity of lysin rendered inactive by the inhibitor varies as a power of the lysin initially present (or, for that matter, of the lysin left free). On the other hand, it is scarcely to be expected that a reaction between two such colloids as saponin and the serum-proteins would be expressible by anything better than an empirical formula.

The effect of varying the concentration of the cell suspension.

All previous investigations have been carried out with reference to suspensions of "standard" strength, but we shall now show that the phenomenon of inhibition by plasma and serum can be completely understood only if we consider what happens when the concentration of the cell suspension is varied.

Consider first what ought to happen if the foregoing account of the inhibition is adequate. Each one of a family of Δ_1, c_1 curves for different values of M, when obtained by using a suspension of a particular strength s_1, will be described by

$$\Delta = \alpha(c_1 - c_{1\infty s_1})^{1/\mu},$$

where $c_{1\infty s_1}$ is the asymptote of the standard curve. If a suspension of strength s_2 is used, and if the foregoing analysis takes account of all the facts, the members of the new family of Δ_1, c_1 curves should be described by

$$\Delta = \alpha(c_1 - c_{1\infty s_2})^{1/\mu},$$

where $c_{1\infty s_2}$ is the asymptote of the standard curve for the s_2 suspension. Thus each member of the second family ought to be the same as the corresponding member of the first family, except that the former is translated upwards or downwards by a distance equal to $(c_{1\infty s_1} - c_{1\infty s_2})$.

Experiment shows, however, that other differences are introduced by varying the strength of the suspension. For example, using a $2x$ standard and a $0{\cdot}5x$ standard suspension, we get the following figures:

	δ	c_1	$c_1 - c_{1\infty}$	Δ
Saponin *plus* 0·16 ml. serum, $2\times$S suspension	20,000	100·0	72·0	31·3
	25,000	80·0	52·0	28·0
	30,000	66·7	38·7	24·8
	38,000	52·6	24·6	21·1
Saponin *plus* 0·16 ml. serum, $0{\cdot}5\times$S suspension	25,000	80·0	55·6	33·1
	30,000	66·7	42·3	30·0
	35,000	57·1	32·7	26·9
	37,000	54·1	29·7	26·3

Analysis of curves such as these shows (*a*) that the Δc_1 curves for the $0{\cdot}5x$ standard suspension make an intercept on the c_1-axis which is less than that made by the curves for the systems containing the $2x$ standard by the amount required by theory, but (*b*) that the value of α is not the same for the two families, although the value of μ is the same. This means that the quantity of lysin apparently inhibited by a particular quantity of serum or plasma depends on the number of cells present in the suspension used for obtaining the time-dilution curves, and that for the same values of c_1 more lysin appears to be inhibited when the strength of the suspension is small than when it is greater. At first this observation puzzled us a great deal, but it can be explained quite easily if we take into account certain other known properties of the systems under consideration. The explanation involves giving up the idea that there is complete irreversibility of the compound formed by the lysin and the plasma-proteins.

We know that when lysin is added to cells in concentration c_1, a quantity of lysin $= \zeta c_1$ is concentrated at the cell surfaces, thus leaving free a quantity $c_1(1-\zeta)$. So far as we know from rather limited observations, ζ is a constant. As lysis goes on, the quantity of "free" lysin decreases, but always in such a way that it is nearly a linear function of c_1 (see Ponder [1932] the section dealing with the "second addition effect"). In any system containing cells, lysin and inhibitor, we have therefore to distinguish between three quantities: the quantity of lysin "absorbed" on the cells, the quantity left free and the quantity inhibited as a result of its interaction with the serum or plasma-proteins. The relation of these quantities will be

	Absorbed	Free	Inhibited
Before cells added	0	c_2	c_3
After cells added	c_4	c_5	c_6

Remembering that $c_1=c_2+c_3=c_4+c_5+c_6$, and that $c_4=\zeta c_5$, and putting, for simplicity's sake, $c_3=\alpha c_2$, we get

	Absorbed	Free	Inhibited
Before cells added	0	$c_1/1+\alpha$	$\alpha c_1/1+\alpha$
After cells added	$\zeta c_1/1+\zeta+\alpha$	$c_1/1+\zeta+\alpha$	$\alpha c_1/1+\zeta+\alpha$

The addition of the cells should therefore result in the reduction of the amount of lysin inhibited from $\alpha c_1/1+\alpha$ to $\alpha c_1/1+\zeta+\alpha$. Clearly the greater ζ the greater the reduction; hence the greater the strength of the suspension used, the smaller will the quantity of lysin inhibited appear, for ζ increases as the number of cells increases. If $c_3=\alpha c_2^{1/\mu}$, no simple solution is possible, but it will be obvious that the effect will be in the same direction, and that the value of μ should be independent of the strength of the suspension. This is just what is found.

This theory appears entirely adequate, but it demands one rather curious condition in the haemolytic system in order that expression (3) be satisfied. When the cells are added to the system containing the plasma or serum, a redistribution of the quantities of lysin absorbed, free and bound to the inhibitor occurs in the way shown above. The course of the ensuing lysis, however, is exactly the same as that in a system containing no inhibitor, but having the lysin present in smaller initial concentration (see expression (3)). In fact, the redistribution having once been effected by the addition of the cells, the lysin bound to the inhibitor behaves as if it were permanently removed from the system. As haemolysis proceeds, the concentration of free lysin may fall, and probably does, but it is not continually replenished by the inhibitor giving up some of its bound lysin, as in a simple reversible system. We do not pretend fully to understand this, but it undoubtedly has a relation to a series of observations which will shortly be published elsewhere, and which shows that the kinetics of haemolysis require to be considered in relation to a sort of "internal" system rather than in relation to the system considered as a homogeneous whole, and that the properties of this "internal" system depend largely on the way in which the lysin is concentrated at the cell surfaces. In such systems, characterised by extreme heterogeneity, the laws for reactions in homogeneous systems certainly do not apply, and we require to seek information regarding their properties by experiment rather than by applying existing theory.

Summary.

This paper is concerned with a re-investigation of the problem of the inhibition of a simple lysin by plasma or serum, using better methods of experiment and analysis than have been used hitherto. The previous conclusion that it is permissible to treat the inhibition as a reaction between lysin and plasma-protein, by means of which the lysin is removed from the system, is confirmed. It is also shown that the quantity of lysin so removed depends on the number of cells present in the system, and this observation is shown to have a simple explanation.

REFERENCES.

Ponder (1923). *Proc. Roy. Soc. Lond.* B **95**, 42.
—— (1925). *Proc. Roy. Soc. Lond.* B **98**, 484.
—— (1932). *Proc. Roy. Soc. Lond.* B **110**, 1.
—— and Yeager (1930). *Proc. Roy. Soc. Lond.* B **106**, 506.

CV. THE EFFECT OF DESICCATED THYROID, IRRADIATED ERGOSTEROL AND AMMONIUM CHLORIDE ON THE EXCRETION OF CALCIUM IN RATS.

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Parhon [1912] observed a marked loss of calcium in rabbits which received lethal doses of thyroid substance by mouth. Aub *et al.* [1929] reported that patients with hyperthyroidism showed an excretion of calcium much greater than that observed on similar diets in hyperparathyroidism or after the administration of acids; he pointed out that the high calcium excretion of hyperthyroidism was unusual in that it occurred chiefly by the intestinal route. Parhon *et al.* [1932] found that demineralisation of the skeleton occurred in dogs during thyroid feeding and was not increased by simultaneous administration of parathyroid extracts. Tibbetts *et al.* [1932] announced that the high faecal excretion of calcium in clinical hyperthyroidism was not influenced by administration of irradiated ergosterol; Watchorn [1930], Pugsley [1932] and others have found however that irradiated ergosterol tends generally to decrease the faecal and increase the urinary excretion of calcium. The present paper reports a study of the effect of desiccated thyroid on the calcium excretion of rats and the influence of irradiated ergosterol[1] thereon, while some experiments on the effect of ammonium chloride acidosis are included for comparison.

Methods.

The experiments were similar in general plan and in the analytical methods used to those described by Pugsley [1932] in a study of the effects of parathyroid extracts and of irradiated ergosterol on the calcium and phosphorus metabolism of rats. Adult male and female rats of Wistar strain, weighing approximately 240 g., were used. The diet, which contained 0·056 % calcium, was given as a constant weighed ration daily, and was not increased during thyroid treatment in spite of the greater appetite of the animals; the weight of unconsumed food was also recorded. Metabolic rates were found by determination of oxygen consumption in a closed-circuit apparatus, similar to that described by Benedict and Macleod [1929], save that the fall of the oxygen reservoir-spirometer is traced on a rotating smoked drum, the tracing serving also as a record of activity. The metabolic rates are expressed as the percentage increase in oxygen consumption over the oxygen consumption of the individual animals determined in the pre-experimental period.

The desiccated thyroid was supplied by Messrs Ingram and Bell, and was found to contain 0·141 % calcium; it was thoroughly mixed with the food each day, so that each rat received 2 grains daily if, as was almost invariably the case, the entire ration was consumed. Irradiated ergosterol (Mead, Johnson & Co.)

[1] The authors are indebted to Mead, Johnson & Co. for the ergosterol used in these experiments.

was suitably diluted with olive oil and given from a pipette in daily doses of 0·1 ml. Ammonium chloride was given daily at the time of feeding by stomach tube, subcutaneous injection at the dosage required having proved highly toxic.

Results.

The effect of administering desiccated thyroid (2 grains daily) is shown in Fig. 1, which is a composite graph representing the average of six similarly

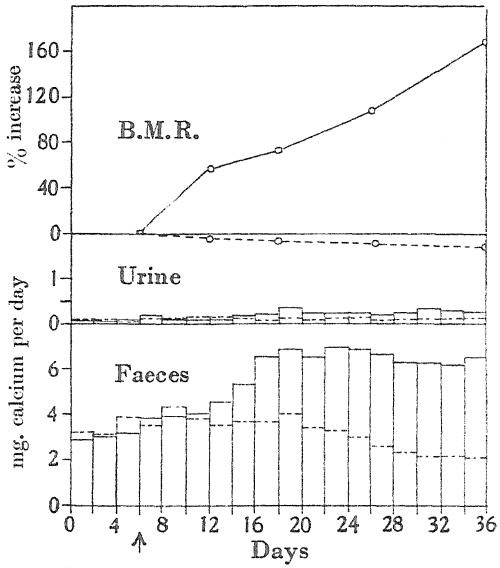

Fig. 1.

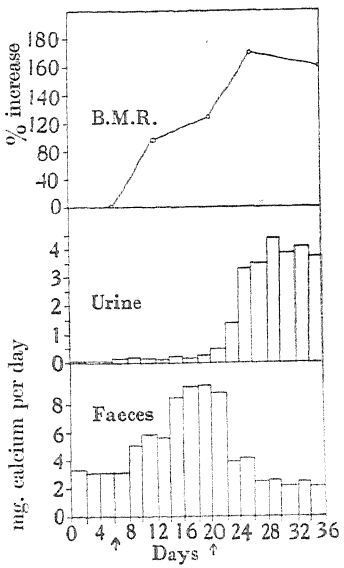

Fig. 2.

Fig. 1. Effect of administering 2 grains desiccated thyroid daily on calcium excretion (average of 6 rats); the dotted line shows the calcium excretion of controls.

Fig. 2. Effect of administering 20,000 units of irradiated ergosterol daily upon the increased calcium excretion induced by thyroid feeding (average of 6 rats). The first arrow indicates the commencement of thyroid treatment, the second the addition of irradiated ergosterol.

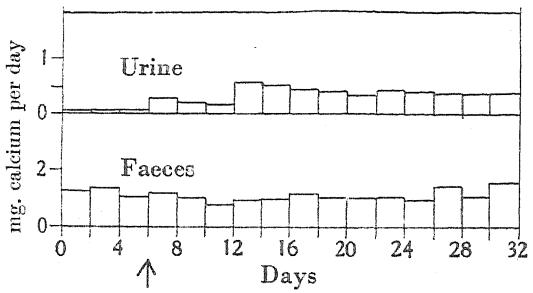

Fig. 3. Effect of administering 125 and (later) 250 mg. ammonium chloride daily on the calcium excretion (average of 6 rats).

treated animals. The rats weighed approximately 250 g. at the beginning of the experiment and lost about 70 g. under thyroid treatment. Thyroid medication began on the sixth day, as indicated by the arrow. The dotted lines in the

figures represent the average behaviour of four untreated control rats, in a simultaneous study.

The administration of thyroid soon leads to a marked increase in the faecal excretion of calcium, which becomes approximately constant at the new level, while the basal metabolic rate (B.M.R.) increases progressively. There is also a decided increase in the urinary excretion of calcium (shown, for clearness, on a larger vertical scale) and the ratio between urinary calcium and faecal calcium, initially very small, does not appear to be greatly altered under thyroid treatment.

Fig. 2 represents another experiment on a series of six rats; thyroid treatment was begun as before on the sixth day, but from the twentieth day (second arrow) the animals also received 20,000 international units of irradiated ergosterol daily by mouth. This dosage has been found in similar experiments on normal rats [Pugsley, 1932] to produce a very marked rise in the urinary calcium excretion with a corresponding decrease in the faecal excretion and without any very pronounced effect on the total calcium balance or the general well-being of the animals. It is clear from the figure that in the present experiments the irradiated ergosterol has again largely diverted the calcium excretion from the faeces to the urine, the total excretion of this element (on practically constant intake) being decreased rather than increased, though still far above the levels observed before thyroid feeding.

Smaller doses of irradiated ergosterol have proved to be less effective. Table I summarises an experiment similar in plan to the last, carried out on another series of six rats. The usual effect of thyroid administration was

Table I. *Effect of administration of 1000 and 10,000 units of irradiated ergosterol daily on the increased calcium excretion induced by thyroid feeding.*

Average of four rats.

Treatment	Day	Urine-Ca mg./day	Faecal Ca mg./day	Ca balance mg./day	Body wt. g.	% increase in O_2 consumption
Control period	2	0·04	4·93	+2·33	224	—
	4	0·06	4·80	+2·76	229	—
	6	0·05	4·59	+2·71	230	0
2 g. desiccated thyroid daily	8	0·04	4·57	+2·63	228	—
	10	0·05	4·60	+1·51	—	—
	12	0·05	5·77	+1·13	221	—
	14	0·03	7·37	−1·40	—	—
	16	0·11	9·25	−3·36	216	—
	18	0·15	10·50	−3·65	—	—
Added 1000 units irradiated ergosterol daily	20	0·11	11·50	−4·61	209	112
	22	0·37	11·50	−3·87	—	—
	24	0·50	12·10	−4·48	—	—
	26	0·57	11·47	−3·79	197	—
	28	0·71	13·67	−5·98	—	—
	30	0·48	12·05	−4·13	188	—
	32	0·74	13·00	−5·34	—	—
10,000 units daily; thyroid continued	34	0·70	13·80	−6·10	180	100
	36	1·22	10·90	−3·72	—	—
	38	1·52	9·17	−2·29	168	—
	40	1·47	9·32	−2·39	—	—
	42	2·47	9·10	−3·17	172	—
	44	2·85	5·58	−0·03	163	—
	46	3·70	4·60	+0·10	—	—
	48	4·24	4·46	−0·30	149	156
	50	3·77	5·62	−0·99	—	—
	52	5·00	3·30	+0·10	—	—
	54	6·72	2·60	−0·92	142	—

observed. The administration of 1000 units of irradiated ergosterol daily, from the twentieth day, may have slightly increased the urinary excretion of calcium but had no discernible effect on the faecal or the total excretions. On the thirty-fourth day the dosage was therefore increased to 10,000 units daily; the table shows that this led in the course of a few days to a definite shift of excretion from faeces to urine, with an unmistakable decline in the total calcium excretion, the negative calcium balance being greatly reduced.

Table II summarises another similar experiment on a series of six rats, in which the dosage selected was 2000 units of irradiated ergosterol daily, from the

Table II. *Effect of administration of 2000 units of irradiated ergosterol daily on the increased calcium excretion induced by thyroid feeding.*

		Average of four rats.				
Treatment	Day	Urine-Ca mg./day	Faecal Ca mg./day	Ca balance mg./day	Body wt. g.	% increase in O_2 consumption
Control period	2	0·04	4·36	+2·60	231	—
	4	0·05	4·40	+3·17	236	—
	6	0·03	3·77	+3·76	238	0
2 g. desiccated thyroid daily	8	0·06	3·30	+3·64	236	—
	10	0·05	3·00	+3·35	—	—
	12	0·04	4·57	+2·11	231	—
	14	0·02	7·20	−1·04	—	—
	16	0·05	7·30	−1·19	223	—
	18	0·07	8·87	−0·82	—	—
Added 2000 units irradiated ergosterol daily	20	0·02	10·25	−2·21	216	79
	22	0·11	10·12	−1·83	—	—
	24	0·13	9·12	−1·01	—	—
	26	0·22	10·30	−2·12	210	—
	28	0·47	10·48	−2·55	—	—
	30	0·54	10·50	−2·65	207	—
	32	0·48	10·51	−2·75	—	—
	34	0·54	10·77	−2·91	206	83
	36	0·67	10·00	−2·27	—	—
	38	0·38	10·00	−1·98	196	—
	40	0·66	10·00	−2·26	—	—
	42	0·24	10·00	−1·84	188	—
	44	0·40	10·90	−2·90	—	—
	46	0·48	10·80	−2·80	165	—
	48	0·45	11·20	−3·25	—	200

twentieth day. Such doses have been found to reduce the faecal excretion of calcium, in normal animals, very decidedly [Pugsley, 1932]; but in the present experiments, although the faecal excretion and negative balance are somewhat lower and the urinary excretion definitely higher than might have been expected in view of the earlier results, there is not convincing evidence that this dose was in any degree effective.

From a series of 12 rats receiving 2 grains of desiccated thyroid daily, blood was collected by the method previously described [Pugsley, 1932] for determination of the serum-calcium by the method of Clark and Collip [1925]. The values obtained fell within the normal range for rats in this colony, the average being 10·3 mg. per 100 ml. (minimum 10·1, maximum 10·4). This agrees with the general opinion [Thomson and Collip, 1932] that there is no simple relation between thyroid function and the level of the serum-calcium.

The effect of ammonium chloride on calcium excretion was studied in six rats, and the results are summarised in Fig. 3. 1 ml. of a 12·5 % solution of the salt was administered daily by stomach tube from the sixth day; as this had little effect, the dose was increased to 2 ml. from the fourteenth day onward

A marked diuresis naturally resulted, and the excretion of calcium in the urine was considerably increased, the faecal excretion being scarcely affected. This observation confirms the view of Aub *et al.* [1929] that the thyroid hormone does not affect calcium metabolism merely by producing an acidosis.

Discussion.

The work of Aub *et al.* [1929] on the effect of the thyroid hormone on calcium excretion has, curiously enough, stimulated little experimental study. We have entirely confirmed his view that the thyroid hormone stimulates calcium excretion and promotes loss of calcium from the body, and that the channel of excretion is the intestine rather than the kidney (a matter in which this effect differs strikingly from the increased calcium excretion produced by parathyroid hormone, by ammonium chloride or by very large doses of irradiated ergosterol). It seems at present futile to speculate on the factors responsible for directing calcium excretion to one channel rather than another, but it should be emphasised that in our experiments with thyroid feeding the amount of calcium lost in the faeces was often considerably greater than the total calcium intake. Our work also supports the contention of Aub that the action of the thyroid hormone is not to produce acidosis or parathyroid stimulation, and moreover does not seem to be directly correlated with the increase in basal metabolic rate. In experiments not reported herein, we have found that the injection of parathyroid extract into rats receiving desiccated thyroid increases the total loss of calcium from the body. The failure of Tibbetts *et al.* [1932] to observe a reduction in faecal calcium excretion and negative calcium balance, as we did, after administration of vitamin D, may perhaps be ascribed to inadequate dosage; Taylor and Weld [1932] obtained results similar to ours in dogs.

Summary.

The administration of 2 grains of desiccated thyroid daily, by mouth, to adult rats, leads to a marked increase in calcium excretion and establishment of a negative calcium balance. The increase is observed in the faecal rather than in the urinary calcium. The serum-calcium is not elevated.

The administration of irradiated ergosterol, in doses of 10,000 international units or more daily by mouth, to rats receiving desiccated thyroid reduces the faecal and increases the urinary excretion of calcium, and partly or wholly corrects the negative calcium balance. Smaller doses are comparatively ineffective.

The administration of 250 mg. ammonium chloride daily, by mouth, to normal adult rats leads to a marked increase in the urinary excretion of calcium, with little influence upon the faecal excretion.

Our thanks are due to Prof. J. B. Collip and Dr D. L. Thomson for their assistance and advice.

REFERENCES.

Aub, Bauer, Heath and Ropes (1929). *J. Clin. Invest.* **7**, 97.
Benedict and Macleod (1929). *J. Nutrition*, **1**, 343.
Clark and Collip (1925). *J. Biol. Chem.* **63**, 461.
Parhon (1912). *Compt. Rend. Soc. Biol.* **72**, 620.
—— Derevici and Derevici (1932). *Compt. Rend. Soc. Biol.* **109**, 1396.
Pugsley (1932). *J. Physiol.* **76**, 315.
Taylor and Weld (1932). *Brit. J. Exp. Path.* **13**, 1109.
Thomson and Collip (1932). *Physiol. Rev.* **12**, 350.
Tibbetts, McLean and Aub (1932). *J. Clin. Invest.* **11**, 1273.
Watchorn (1930). *Biochem. J.* **24**, 631, 1560.

CVI. SOME PROPERTIES OF THE POLYSACCHARIDE COMPLEX EXTRACTED FROM A MARINE ALGA, *CHONDRUS CRISPUS*.

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THE chemical nature of the water-soluble polysaccharide constituent of *Chondrus crispus* has previously received considerable attention. The interest manifested in it is attributable to its possession of marked gelatinising power. On account of this property it lends itself to a variety of uses. Unfortunately, the results to be found in the literature are based on variable methods of extraction, many of which are not very rational. The primary aim of the present work was, therefore, the preparation of a pure standard extract, so that the data obtained might be significant and of general application. The establishment of a definite criterion of purity is no simple matter, as the material is non-crystallisable and decomposes at very low temperatures. However, despite this, a reasonably pure standard extract has been obtained and from it have been prepared salts which give evidence of an even higher degree of purity.

HISTORICAL.

Investigation of this particular species, *Chondrus crispus*, and the material extracted from it in aqueous solution, dates from the work of Flückiger and Obermayer [1868]. In Abderhalden's *Biochemisches Handlexikon* [1911] there appeared the statement that "the pure mucilage of carrageen[1] corresponds to the formula $C_6H_{10}O_5$." The evidence on which this formula is based was not given. Though it is conceivable that it might represent the carbohydrate moiety, this formula is quite inadequate for the complete substance, since it takes no account of the 20 % ash content. There is also present some nitrogen which, until proven to be an impurity, cannot be disregarded. Apart from the fact that it is largely carbohydrate, very little is definitely known of the nature of the material in *Chondrus crispus* which is responsible for gelation. Haas [1921] advanced the theory that it is a colloidal electrolyte—an ethereal sulphate of calcium, the general structure of which might be represented by formula I.

$R(OSO_2O)_2Ca$ I $\qquad$ $Ca(OSO_2OR)_2$ II

Harwood [1923], making use of physico-chemical methods, finds the evidence contradictory and points out that without further experimental work it is impossible to choose between the alternative formulae, I and II. He appears, however, inclined to agree with Haas that I is more acceptable.

[1] The aqueous extract of *Chondrus* is commonly referred to in this way; the plant itself is also frequently called carrageen or Irish Moss.

On the other hand Haas's assumption of this disulphate ester has been criticised by Nelson and Cretcher [1931], on the grounds that the analytical data are insufficient to indicate this composition of the esters and to preclude the presence of polymerides of monosulphate esters.

Even should one of these ultimately be found correct, for the present one serves as well as the other as a working hypothesis. It is not impossible that both types may be present in the extract.

According to Haas, the calcium is ionised, but the sulphate ion is masked by its combination with the carbohydrate part of the molecule. On heating, this type of compound breaks down thus:

$$R(OSO_2O)_2Ca + 2H_2O = R(OH)_2 + H_2SO_4 + CaSO_4 \quad \text{.....(i).}$$

Later Haas altered his procedure and instead of using the dried extract with which he had previously worked [Haas and Hill, 1921] he prepared two fractions. One was a cold water extract (C.E.) and the other a hot water extract (H.E.) of the plants from which, presumably, all the C.E. had been removed.

The occurrence of a similar calcium sulphate-carbohydrate compound in agar-agar was reported in the same year [Neuberg and Ohle, 1921]. Haas and Russell-Wells [1923] made a fairly extensive study of a number of seaweeds. They found that in nearly all species having high ash contents the presence of an ethereal sulphate in the plants was indicated.

The evidence on the strength of which the ethereal sulphate formula was assigned is as follows:

(1) The calcium is freely ionised and can be quantitatively precipitated by ammonium oxalate even before hydrolysis.

(2) The sulphate content of the ash is high.

(3) The sulphate cannot be precipitated from the solution until after hydrolysis.

(4) The amount of the sulphate in the ash is only half of that in the hydrolysed solution. This loss of half the sulphate on ashing is attributed to its escape as sulphuric acid, according to equation (i).

Experiments with algae other than *Chondrus crispus* showed variations in the 2:1 ratio of "sulphate by hydrolysis" to "sulphate in ash." Nevertheless, Haas believes it permissible to assume, when the amount in the hydrolysed solution is greatly in excess of that in the ash, that the sulphate occurred in the plant in an ethereal linkage of the type described. The discrepancies from the 2:1 ratio are explained, in the case of higher ash sulphate, as due to the retention by an alkaline ash of some of the sulphuric acid which should normally have escaped. In cases where the sulphate in the ash is less than the theoretical, the loss is attributed to the presence of an ammonium salt, which would leave no ash.

Experimental.

Preparation and analysis of samples.

Preparation. From a number of methods of extraction and precipitation investigated, the following was finally adopted as being the most satisfactory method of obtaining a standard preparation.

20 g. of *Chondrus crispus* plants are washed with cold distilled water until free from chlorides. (Three or four washings are usually sufficient.) The washed *Chondrus* is then transferred to a flask with a litre of distilled water, and heated on a boiling water-bath for 5 or 6 hours. The resultant viscous mixture is centrifuged and the clear liquid filtered through a Chardin filter-paper on a Büchner funnel encased with a hot water jacket. It is necessary to change the filter-paper

frequently. The filtrate, kept in constant motion by stirring, is evaporated on a water-bath to approximately 300 ml. It is then poured, slowly and with stirring, into one litre of 95 % alcohol. A stringy, fibrous material is precipitated, which is removed as soon as formed, allowed to drain through cheese-cloth and finally transferred to 250 ml. of 95 % alcohol, in which it remains until the next day. It is then transferred daily from one to the next of the following washes: (1) and (2) 200 ml. absolute alcohol; (3) and (4) 150 ml. anhydrous ether. The precipitate is then dried in a vacuum-desiccator for a day and finally ground to a fine powder. It is dried in air for at least 6 hours at approximately 80° before being used. If the temperature is allowed to rise above this for any length of time charring occurs. The product is a creamy-white, non-crystalline powder, soluble in both cold and hot water and forming in 2 % solution a firm gel.

Ash. The ash content of the extract does not represent simply salt content but combined inorganic material. It does not, as will be seen later, represent all of this, but it may be expected to represent approximately the same percentage of the combined inorganic material in all cases, since the percentage lost depends on the original structure. Ash determinations of a number of extracts prepared from different collections are given in Table II.

It will be observed that the differences in ash content are slight, in spite of the fact that these extracts are made from plants collected at different places and in different seasons (Table I).

Table I. *Identification of samples.*

Sample	Place of collection	Month of collection
S	South Shore, P.E.I.	August
N	Souris, P.E.I.	,,
H	Halifax, N.S. (Pt. Pleasant)	February
T	,, (McNab's Island)	August
D	,, (Devil's Island)	June
P	Peggy's Cove, N.S.	August
C	Cavendish, P.E.I.	,,
F	Details of collection missing. Extract made without any washes of absolute alcohol	

The standard extract used in the present investigations was prepared from sample C.

Table II. *Percentage of ash in different extracts.*

Sample	% ash (dry weight)	Sample	% ash (dry weight)
F	17·30	S	19·11
D	19·32	N	18·43
T	19·46	H	16·36
P	19·74	C	19·44

As previously noted, many investigators regard the ash content of marine algae as practically pure calcium sulphate. The fact that the SO_4 content of the ash (see Table VI) averages only 58 % negatives this assumption. (SO_4 calculated for calcium sulphate is 70·34 %.)

The ash was found to contain varying quantities of potassium up to 12 % (Table VI). Russell-Wells [1922] reports that the ash of her extract contained sulphate, calcium, magnesium and small quantities of sodium and potassium, with traces of iron. The extract, therefore, probably contains a calcium ethereal sulphate, with which there are associated analogous salts of other metals. There is found also some phosphate which was not originally precipitable as the phosphate ion, and which may be assumed to have been present in an organic combination.

Sulphate. During the present investigation, sulphate (SO_4) was determined in three different preparations: (1) in hydrolysed samples; (2) in oxidised

samples; (3) in the ash. In each case the sulphate was precipitated by means of barium chloride, ignited and weighed.

Hydrolysis. In order to ascertain when hydrolysis was complete, a number of methods have been tried. The results (Table III) show that the sulphate is much more resistant to hydrolysis by alkali than by acid. It is evident also that all the sulphate is freed by boiling directly, for 5 hours, in 5 % hydrochloric acid, whereas even after 13 hours on the water-bath nearly 2 % still remains combined. This latter fact might be taken to indicate that the sulphur occurs in more than one type of combination.

Table III. *Sulphate by hydrolysis of extract.*

Method of hydrolysis	Time (hrs.)	% sulphate (SO_4)
Sample C:		
Boiled directly with 5 % hydrochloric acid	5	27·66
,, ,,	6	27·53
,, ,,	13	27·44
On water-bath with 5 % hydrochloric acid	6	21·62
,, ,,	13	25·62
Boiled directly with 5 % sodium hydroxide	6	13·32
Sample H:		
Boiled directly with 5 % hydrochloric acid	6	25·18
Boiled on sand-bath with 40 % hydrochloric acid*	6	25·38

* [Haas and Russell-Wells, 1923.]

Oxidation. The oxidation was accomplished by fusion with a hydroxide-nitrate (8:1) mixture. The melt was dissolved and acidified with hydrochloric acid, after which the sulphate was precipitated with barium chloride (Table IV).

Table IV. *Sulphur in extract after oxidation.*

Sample*	% sulphate (SO_4)	Sample*	% sulphate (SO_4)
I	29·04	W	26·70
	28·08		27·72
II	27·60	A	28·20
	29·07		29·28
III (*a*)	28·56	3X	30·24
	28·95		30·09
III (*b*)	28·02	5X	28·53
	27·96		
H	28·95		
	29·31		

* Differing symbols here indicate slight variations in the standard method of extraction, made with another purpose in view, but all are from the same collection of plants, namely sample C.

The sulphate after oxidation was found to average 28·38 %. The difference between this figure and that obtained by the hydrolysis, being very little outside the range of experimental error, seemed insufficient to warrant the assumption of any appreciable amount of unoxidised sulphur.

Ash. From the ash of the standard sample C, the sulphate was found to be 11·54 %, which figure does not satisfy equation (i), since in the present case only 35 % of the sulphate has been retained, rather than 50 %. Russell-Wells [1922] reports more than 50 % of the sulphate to be lost on ashing and attributes the excess loss to the presence in the original of some ammonium ethereal

sulphate, the sulphur of which would of course be completely lost on ashing. In the present case, however, the excess loss is too great to be explained in this manner, because the amount of nitrogen present would be sufficient to account for only 1 % of sulphate at the most (unpublished data).

As noted above, the ratio of "sulphate in hydrolysate" to "sulphate in ash" was found to be more nearly 3:1 than the 2:1 theoretically required by Haas's formula. This suggests the possibility that there might occur in the molecule three sulphate groups, only one of which is retained in the ash. An acid salt of the structure shown in the following equation would explain the observed results:

$$\underset{\text{III}}{HOSO_2O.R(OSO_2O)_2Ca} + 3H_2O = R(OH)_3 + 2H_2SO_4 + CaSO_4 \qquad \text{.....(ii).}$$

The sulphate retained in the ash of a compound such as III is one-third of that in the original.

Nelson and Cretcher [1931] found, in their analysis of the carbohydrate acid sulphate of *Macrocystis pyrifera*, that the percentage of ash obtained without oxidation was somewhat lower than when the sample was ashed in the presence of sulphuric acid. This they believe to be due, not to the retention of unoxidised sulphur in the latter case, but to the reduction of some sulphate in the former.

In order to determine whether or not the sulphate being lost here, in excess of that normally escaping as sulphuric acid, was due to this cause, approximately 0·2 g. of the sample was ashed in an electric muffle furnace, after having been moistened with nitric acid. A sample was ashed in the same manner with sodium carbonate (Table V). These figures show plainly that the sulphate loss

Table V. *Percentage of ash and sulphate in extract of sample C after different methods of ashing.*

Method	% ash (dry weight)	% SO_4 calc. to original extract
Simple incineration	19·44	11·54
Ashed with nitric acid	19·33	11·67
Ashed with sodium carbonate	—	28·49

experienced on ashing cannot be attributed to reduction of the sulphate. That the sulphate obtained after hydrolysis is the true sulphate value is shown by the agreement between it and that obtained after ashing with sodium carbonate, as well as that from the fusion with hydroxide-nitrate mixture (see Table III).

Preparation of salts.

Potassium salts. A 1 % solution of the extract was dialysed in collodion membranes[1] against frequently replaced potassium chloride until the dialysate gave a negative test for calcium (5 % potassium chloride was used at first, then 2·5 %). The dialysed material was then tested to make sure that no calcium had been retained. Dialysis was then continued against distilled water, until the excess of potassium chloride was removed. It was noticed that shortly after dialysis against the potassium chloride began, the extract became opaque and soon set to a gel. During continued dialysis against distilled water the material gradually liquefied.

[1] These membranes were made with the following collodion solution: 5 g. pyroxylin, 25 ml. absolute alcohol, 65 ml. anhydrous ether, 10 ml. ethylene glycol. Membranes were allowed to dry 25 to 30 minutes before being filled with water [Pierce, 1927].

The material, now free from calcium, is concentrated on the water-bath, precipitated and dehydrated according to the standard method. The product, a fine, pure white powder, is the potassium salt or salts resulting from replacement of the calcium and other cations of the water-soluble salts which are present *in situ* in the plant.

This material was found to have an actual ash content of 25·56 %. In appearance, the ash obtained from the original extract is frequently a dirty fawn colour and fused, while that of the potassium salts is snowy white and powdery. This ash contained K, 45·18; SO_4, 55·14 %. K_2SO_4 requires K, 44·89; SO_4, 55·11 %.

Now the plant *Chondrus* itself contains[1] 4·58 %, and the standard extract 2·22 % of potassium. On the other hand, the material obtained by dialysis against the potassium chloride solution contains only 0·069 % of calcium. Further evidence of the purity of this product is found in the absence from it of any trace of phosphorus. Positive tests for phosphorus were given by all other extracts, except that in the preparation of which the plants had been washed for 3 days in running water before the extract was made. When the period of dialysis was prolonged the resultant preparations showed that most of the cations of the extract were replaced by potassium during the first few hours, and that subsequent dialysis affected but slightly the total amount of potassium introduced.

Ammonium salts. Ammonium salts were prepared in a similar manner to the potassium salts, except that the final solution of the ammonium salts was not precipitated but was evaporated to dryness. Complete dialysis in this case required from 2 to 3 weeks, sometimes more. This ammonium salt gives less than 1 % of ash, whereas that which Russell-Wells [1922] prepared by another method yielded 5 % ash. The original sulphate in this material was found by hydrolysis to be 28·49 %.

Calcium salts. The potassium salt prepared as described above is obviously a more nearly pure material than the original extract. It was determined therefore to regenerate the calcium salt from it by dialysing the potassium derivative against calcium chloride (a 2·5 % solution was used) until no potassium could be detected in the dialysate. This point was reached in about 48 hours, after which the excess of calcium chloride was removed by dialysis against distilled water. The solution of the "regenerated" calcium salt was then evaporated, precipitated and dehydrated as in the standard preparation.

The material obtained differed in appearance from both the original and the potassium salt and decomposed much more readily even than the original.

The ash values of the original and the "regenerated" calcium salt did not differ greatly. The sulphate and calcium in the ash, however, were found to be very different. Figures are given in Table VI, which includes the potassium salt, for the sake of comparison, as well as the potassium figures for all three.

Table VI clearly shows to be erroneous the assumption that the original extract is a calcium salt. For one thing its ash contains more potassium than calcium. The difference between the calcium contents of the original extract and of the "regenerated" calcium salt is very great. The simultaneous decrease in potassium is not sufficient to account for this, nor is the increase which would occur by substitution of calcium in place of the hydrogen in formula III sufficient to account for the difference. The presence of still other cations is thus

[1] This figure is for a different collection of plants from that on which the results presented here were obtained.

Table VI. *Comparison of ash and sulphate in prepared salts with standard extract.*

	% ash	% SO_4 in ash	% SO_4 in original calc. from ash	% SO_4 in original by hydrolysis	% calcium in ash	% calcium in original salt calc. from ash	% potassium in ash	% potassium in original salt calc. from ash
Original extract	18·67	58·44	10·91	26·82	2·88	0·52	11·99	2·22
	19·14	58·74	11·54	27·51	3·68	0·68	—	—
Regenerated calcium salt	20·96	68·72	14·40	29·06	32·22	6·75*	0·76	0·16
Potassium salt	25·73	54·47	14·05	27·32	0·28	0·07	45·42	11·70
	25·26	55·14	13·94	28·00	—	—	—	—
Ammonium	0·60	—	—	28·49	—	—	—	—

* Calcium by hydrolysis in this sample was 6·56 %.

indicated. Further, the percentages of calcium and sulphate in the ash of the "regenerated" calcium salt approximate to those of pure calcium sulphate (Ca, 29·66; SO_4, 70·34 %), while they differ markedly from those of the original extract.

It will be noted from Table VI that in the potassium salt the theoretical 50 % of sulphate is lost on ashing. If the cations of a substance such as that represented in formula III were replaced by dialysis against potassium chloride, a neutral potassium salt (IV) would be obtained. This, on ashing, would undergo the following changes:

$$\underset{\text{IV}}{2R(OSO_2OK)_3} + 6H_2O = 3K_2SO_4 + 3H_2SO_4 + 2R(OH)_3 \qquad \text{.....(iii).}$$

It will be seen that here one-half the sulphate is converted into sulphuric acid and lost, as was actually found to be the case with the prepared potassium salt. This fact, together with the already noted absence of calcium and phosphate and the purity of the ash, leads to the conclusion that this preparation is a true ethereal sulphate. In the "regenerated" calcium salt also a 2:1 ratio of "sulphate from hydrolysis" to "sulphate from ash" is established. The original extract, being a mixture of sulphates, the ratio of "hydrolysis sulphate" to "ash sulphate" in it has not the same significance.

Further evidence that the extract of *Chondrus crispus* cannot rightly be regarded as a pure chemical compound was obtained from an examination of

Table VII. *Comparison of fractions with standard extract.*

Tests	C.E.	H.E.	Standard
50 % alcohol	No ppt.	Good ppt.	Good ppt.
Molisch	+	+	+
Biuret	−	−	−
Reduction	−	−	−
Reduction after hydrolysis	+	+	+
Potassium	+	− ?	+
Phosphate	+	−	+
Chloride	None visible	− ?	− ?
Calcium	?	+ + +	+ +
Sulphate	− ?	−	−
Sulphate after hydrolysis	+	+	+
Ash	27·43 %	17·24 %	19·47 %
	26·43	17·35	19·42
	27·49	16·80	
Nitrogen	0·32	0·16	0·24
	0·30	0·15	0·24

the fractions described by Haas [1921]. Extracts were made according to his method of separation and compared with one another and with the standard extract, as previously described. Instead of being simply evaporated to dryness, however, the fractions were precipitated and dehydrated in the same manner as the standard preparation.

Solutions of the two fractions differ greatly in appearance; the H.E. is almost water clear, while the C.E. has a yellowish colour and is very opaque, making it difficult to distinguish precipitates in it. In appearance, as in other properties, the standard is intermediate between the C.E. and H.E. which might be expected, as it is in effect a mixture of the two, containing little C.E. and much H.E. The marked difference in ash content of C.E. and H.E. might be attributed to salts adherent to the plant and taken into solution along with the polysaccharide complex. To obviate this possibility the method of fractionation was modified in the following way. A suspension of the finely ground plants was made, introduced into collodion membranes and dialysed against frequent changes of distilled water. The first dialysates were tested and found to contain chloride, sulphate, calcium and potassium ions. The absence of the chloride ion—because it is the most abundant in sea-water and is detectable longer in the dialysate—was taken to signify that all the salts had been removed. The dialysed suspension was filtered through Chardin filter-paper on a Büchner funnel. The filtrate, which from this point was treated according to the standard method of preparation, yielded the "dialysed cold extract" (D.C.E.) which was free from adherent salts. The residue was then extracted with water on a boiling water-bath for 5 hours. From the filtrate of this extract was obtained, by precipitation and dehydration as before, the "dialysed hot extract" (D.H.E.). It was now possible to make a comparison of the ash material actually combined in the two fractions with that combined in the standard extract.

Table VIII. *Comparison of ash and sulphate content of fractions with standard extract.*

Sample	% ash (dry weight)	% SO_4 in ash	% SO_4 in original salt calculated from ash	% SO_4 in original salt by hydrolysis
Standard	19·42	58·74	11·54	27·51
D.C.E.	21·47	59·36	12·75	28·79
D.H.E.	17·63	60·90	10·74	27·83
H.E.	17·13	63·34	10·85	27·03
C.E.	27·12	—	—	—

Assuming that the difference between the ash of the C.E. and D.C.E. is due to free salts, there still remains a 4 % difference between the ash contents of the dialysed fractions (D.C.E. and D.H.E.). The ash content of the standard is intermediate between the fractions, as a mixture of the two would be. The ash of the D.C.E. contains more phosphorus than that of the D.H.E., which, of course, would help to increase the percentage of ash in the former.

A consideration of the sulphate content of the fractions may mean little or nothing, as the differences recorded are very small.

The original sulphate obtained both by hydrolysis and by calculation from the ash sulphate is higher in the D.C.E. The percentage of sulphate in the ash of the D.C.E. might be depressed somewhat by the presence there of phosphate. The difference in the "hydrolysis sulphate" between the fractions becomes more pronounced when the D.C.E. is contrasted with the H.E. instead of the D.H.E., because the H.E. contains none of the C.E., while the D.H.E. contains some

of it. In respect to sulphate content, therefore, as well as ash, the standard preparation would appear to fill an intermediate position.

It should be noticed, from Table VIII, that the proportion of "hydrolysis SO_4" to "ash SO_4" coincides no better with the 2:1 ratio in the case of the fractions, than it does in the standard as here prepared.

Comparison with agar-agar.

Carrageen is frequently compared with agar-agar. Both are products of marine algae, and possess a common physical characteristic of importance, namely the power to gel in aqueous solution. As the method of preparation of most commercial agars does not eliminate nearly all the impurities, a solution of agar was subjected to the precipitation and dehydration procedure described, before being compared with the standard extract of *Chondrus*. Table IX affords a comparison of the purified and unpurified agar with the standard extract of *Chondrus*.

Table IX. *Comparison of agar with standard extract of* Chondrus.

Tests	Unpurified agar	Purified agar	Standard *Chondrus* extract
Appearance of aqueous solution	Yellowish opaque	Bluish-white opaque	Colourless clear
Molisch	+	+	+
Reduction	?	?	−
Reduction after hydrolysis	+	+	+
Biuret	−	−	−
Calcium	+	+	+ +
Potassium	−	−	+
Chloride	+	+ ?	−
Phosphate	−	−	+
Sulphate	+	?	−
Sulphate after hydrolysis	+	−	+
Ash	4·44 %	2·17 %	19·11 %
Nitrogen	2·51	0·07	0·24

Though it appears from this table that there is no sulphate in the purified agar, this is contrary to conclusions of the majority of investigators [Samec and Ssajevic, 1921; Fairbrother and Mastin, 1923], and as it is the result of a single observation no great importance is attached to it. Even the unpurified agar gives a sulphate test only after hydrolysis of a concentrated solution, or in the ash. However, the basic structure of agar cannot include an ethereal sulphate group, as it does in the case of the *Chondrus* extract. Even supposing the whole ash were composed of sulphates, it would be sufficient to account for the existence of only a small fraction of the original material as an ethereal sulphate similar to that present in the *Chondrus* extract, unless indeed this were an ethereal ammonium sulphate. That it could have been such is precluded by the small amount of nitrogen present in agar. We seem, therefore, to be led to the conclusion that though agar contains a small quantity of ethereal sulphates, they are not such essential constituents as in *Chondrus*. If, on the other hand, it is assumed that the structure of agar is based on the ethereal sulphate linkage, it follows from the difference in ash content that the carbohydrate group is ten times as complex as that in *Chondrus* extract.

Discussion.

The data here presented afford evidence that the material extracted by water from *Chondrus crispus* is not a single substance, but a mixture of several, neither the whole nature nor the number of which is known.

The fractionation suggested by Haas [1921] appears artificial, as neither of the fractions is any nearer being a pure chemical compound than is the standard extract. Russell-Wells [1922] says, "the similarity of oxidation products of both (fractions), points to both consisting of substances belonging to the same group of compounds, but there is not evidence enough to show whether the differences are due to different bodies, or mixtures in different proportions of the same substances. On the whole the former of these alternatives appears the most likely." From the findings here presented, however, it seems more rational to regard the fractions as mixtures of the same or very closely allied substances, in different proportions and to suppose that in addition to the common components, each fraction may contain some substance or substances which do not occur in the other. Thus the C.E. contains inorganic and simple organic materials, *e.g.* phosphates, from which the H.E. is free. It is quite probable that some of the more complex constituents of the H.E. are entirely lacking from the C.E.

The evidence for the purity of salts prepared from the standard extract, on the other hand, is clear cut. In the case of the potassium salt, for instance, there is: (1) an agreement between the percentages of potassium and sulphate in the ash with those of pure potassium sulphate; and (2) an agreement of the sulphate content of the salt and its ash with the theoretical 2:1 ratio (Table VI). Furthermore (3) the removal of the phosphate ion which was present in the standard extract suggests that other impurities also have been removed.

If the potassium preparation is not a single salt, it can only be a mixture of ethereal sulphates of potassium, which are very similar in structure and differ only in complexity. The variations, being such as not to interfere with the agreements noted, must be either in the nature or the complexity of the carbohydrate radical with which the potassium sulphate is united, or in the number of potassium sulphate groups united with the (same) carbohydrate radical.

It has been determined that the sulphate forms 28 % of the whole potassium salt. On the basis of formula V, $R(OSO_2OK)_2$, the molecular weight would be roughly 685, or that of the carbohydrate radical 415. Based on formula IV, $R(OSO_2OK)_3$, the molecular weight would be approximately 1030, or that of the carbohydrate fraction 625. The properties of the material, however, are such as to suggest that the molecular weight is even greater than this. Therefore, one of the following formulae probably represents its constitution more correctly:

$$[R(OSO_2OK)_2]_n \quad \text{or} \quad [R(OSO_2OK)_3]_n$$
VI VII

Since the total ash of this preparation can be accounted for it should prove a very satisfactory material for use in investigating the carbohydrate fraction of the complex.

Summary.

A method which yields a standard and relatively pure substance is described for the preparation of the gelatinising constituent of *Chondrus crispus*. This material is largely carbohydrate in nature but has an ash content equivalent to approximately 20 % of its dry weight.

The extract previously described as an ethereal sulphate of calcium is now shown to be a mixture of sulphates, together with other substances, for example, phosphates.

The potassium salts of the ethereal sulphates which occur *in situ* have been prepared, and the preparation found to be superior in purity to the original extract.

The potassium of this preparation was then completely replaced by calcium and the differences between these "regenerated" calcium salts and the original extract determined. The results invalidate the assumption that the original is solely, or even chiefly, an ethereal sulphate of calcium.

The ammonium salts also were prepared and are compared with those of potassium and calcium and with the original extract.

It was found that more sulphate was lost on ashing the standard extract than corresponded to the theoretical loss, on the basis of the formula proposed by Haas [1921]. This leads to the suggestion of a modified formula—that of an acid salt—which more closely fits the facts. Such an acid salt would, by the method used in the preparation of the potassium, calcium and ammonium salts, be changed into a neutral salt. The sulphate loss on the ashing of these prepared salts corresponds with the theoretical.

The *Chondrus* extract is compared with a purified sample of agar-agar, to which it is similar in physical properties and origin.

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REFERENCES.

Abderhalden (1911). *Biochemisches Handlexicon*, **2**, 75.
Fairbrother and Mastin (1923). *J. Chem. Soc.* **123**, 1412.
Flückiger and Obermayer (1868). *Repert. Pharm.* 380.
Haas (1921). *Biochem. J.* **15**, 469.
—— and Hill (1921). *Ann. Appl. Biol.* **7**, 352.
—— and Russell-Wells (1923). *Biochem. J.* **17**, 696.
Harwood (1923). *J. Chem. Soc.* **123**, 2254.
Nelson and Cretcher (1931). *J. Biol. Chem.* **94**, 147.
Neuberg and Ohle (1921). *Biochem. Z.* **125**, 311.
Pierce (1927). *J. Biol. Chem.* **75**, 795.
Russell-Wells (1922). *Biochem. J.* **16**, 578.
Samec and Ssajevic (1921). *Compt. Rend. Acad. Sci.* **173**, 1474.

CVII. ON THE PHYSIOLOGICAL ACTION OF ASCORBIC ACID AND SOME RELATED COMPOUNDS.

By VICTOR DEMOLE.

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(*Received March 24th, 1934.*)

THE biochemical action of *l*-ascorbic acid has hitherto been studied principally on the scorbutic guinea-pig. Only recently Harris *et al.* [1933] investigated the excretion of ascorbic acid in the human subject and found that with a normal intake of vitamin C about 30 mg. of ascorbic acid can be determined by dichlorophenolindophenol titration in the daily output of urine. The excretion is temporarily increased when large doses of vitamin C (150 ml. lemon juice corresponding to about 150 mg. of ascorbic acid are given). It was also found by van Eekelen *et al.* [1933] that with a normal diet about 25 mg. of ascorbic acid are excreted daily in the urine.

In order to determine if, as in the case of vitamins A and D, symptoms of hypervitaminosis occur after the administration of large quantities of ascorbic acid, the present author has investigated the tolerance of various laboratory animals (cold and warm blooded). The vitamin was administered *per os*, subcutaneously, intraperitoneally and intravenously, using an aqueous solution of ascorbic acid which was neutralised with sodium hydroxide shortly before use. In the following table the maximum single doses given to the various animals are summarised.

Animal	Weight g.	Dosage in g./kg. Oral	Subcutaneous	Intraperitoneal	Intravenous
Axolotl	15–30	—	5	5	—
Frog	30–40	—	5	5	—
Mouse	12–20	5	5	2	1
Rat	120	5	2·5	—	1
Guinea-pig	250	5	1	—	0·5
Rabbit	2500	2	1	1	1
Cat	2500	1	0·5	—	0·5
Dog	8000	0·5	0·2	—	0·2

The doses were well tolerated in every case and no symptoms of hypervitaminosis were observed. Only in the mouse after oral administration was a brief laxative effect observed, this being probably caused by the relatively large quantities of salt administered. The body temperature of dogs and rabbits was not influenced for 24 hours after the injection. The Ca and P of the blood were not altered.

Ascorbic acid was also very well tolerated when administered over a long period. Guinea-pigs maintained on a normal diet received orally, subcutaneously or intravenously for 6 days 0·4–2·5 g. ascorbic acid per kg. daily (total dose 2·4–12 g./kg.). Under similar conditions mice were given 0·5–1 g./kg. for 7 days (a total of 3·5–7 g./kg.). The animals remained completely normal during the experiment and for 14 days thereafter. They showed no difference in appetite,

increase of weight and general behaviour from controls receiving the same amounts of the biologically inactive galacturonic acid. Histological investigation of various organs (kidney, pancreas, liver, heart and lungs) showed no definite changes.

As the minimum curative and prophylactic dose of ascorbic acid for the guinea-pig is 0·5–1 mg. daily and in the above experiments 2·5 g./kg. daily were administered, it is evident that the animals tolerate 500–1000 times the therapeutic dose of ascorbic acid daily without ill effects; the average weight of the guinea-pig being taken as 250 g. Experiments with a still higher dosage seem unnecessary as such is unlikely to occur in practice.

The reducing capacity of the urine for Fehling's solution or iodine in potassium iodide was definitely increased after administration of ascorbic acid. After intravenous or subcutaneous injection of 1–2 g./kg. of ascorbic acid to mice or rabbits, or after oral administration of 3–5 g./kg. to guinea-pigs, the urine obtained an hour later gave with Fehling's solution a pronounced precipitate of cuprous oxide. After 2–4 hours the reducing capacity reached a maximum and subsequently decreased fairly rapidly. After 20–24 hours the excretion was practically ended.

Method.

A dog weighing 9·6 kg. received 1 g. ascorbic acid, corresponding to approximately 0·1 g./kg. The urine was removed by catheter at intervals and the reducing capacity of the various fractions determined iodimetrically. The first fraction of urine (11 ml.), taken after 1 hour, showed a reducing capacity corresponding to a total amount of 0·153 g. ascorbic acid. In the second fraction (27 ml.), taken 3 hours after the injection, the reducing capacity corresponded to 0·377 g. ascorbic acid. The third fraction (27 ml.), taken after 5 hours, contained only 0·11 g. of ascorbic acid. The fourth fraction (100 ml.), taken after 8 hours, contained a total of only 0·057 g. of ascorbic acid. The reducing capacity of later fractions of urine showed normal values. The total excretion of ascorbic acid estimated iodimetrically was 0·701 g., *i.e.* about 75 % of the amount administered. The highest concentration of ascorbic acid (1·4 %) occurred in the second fraction of urine (1–3 hours after the injection). When still larger amounts of ascorbic acid are injected subcutaneously the concentration of ascorbic acid in the urine is increased still further and the excretion is continued for over 20 hours. The observed increase in the reducing capacity of the urine is undoubtedly due to an excretion of ascorbic acid because scorbutic guinea-pigs are cured by a daily dose of 0·2–1 ml. of this urine in 4–8 days.

The ascorbic acid in the circulation also increases the reducing capacity of the blood. After intravenous or subcutaneous injection of 0·4–0·5 g./kg. ascorbic acid to rabbits the apparent values for blood-sugar correspond to 140–160 mg./100 ml., the increase being only temporary. After one or two hours the blood-sugar values have again reached the normal of 110–120 mg./100 ml.

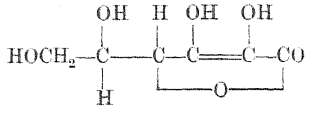
That this increased reducing capacity is not due to a mobilisation of glucose in the blood is proved by the fact that it is impossible to cure the convulsions of a hypoglycaemic rabbit by repeated injections of 0·1 g./kg. ascorbic acid intravenously or subcutaneously.

The action of related compounds.

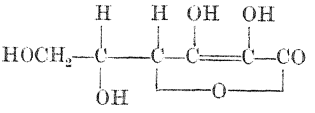
The researches of Reichstein *et al.* [1933, 1, 2; 1934] made it possible to investigate the antiscorbutic action of synthetic *l*-ascorbic acid and some of its isomerides and homologues. The complete identity in biological action of

synthetic *l*-ascorbic acid with the natural product from paprica was established, both when the synthesis was made from xylose and from sorbose as starting material. The isomerides and homologues investigated are shown below (in the case of the heptonic acids the lactone formulae are not given owing to the uncertainty as to whether a five- or six-ring is present):

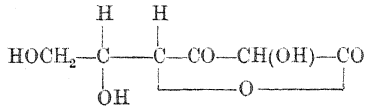
l-Ascorbic acid
Vitamin C



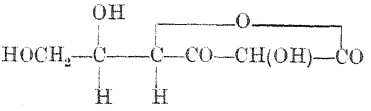
d-Ascorbic acid



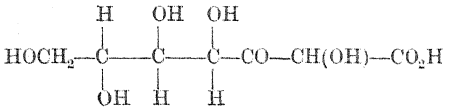
d-Erythro-3-ketohexonic acid



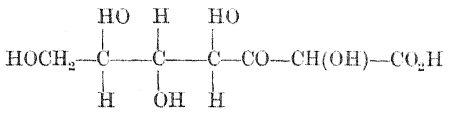
l-Erythro-3-ketohexonic acid



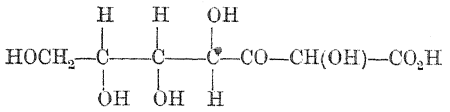
d-Lyxo-3-ketoheptonic acid



l-Xylo-3-ketoheptonic acid



d-Arabo-3-ketohexonic acid



The *d*-erythro-3-ketohexonic acid was found to have about 1/20 of the activity of *l*-ascorbic acid, in agreement with the result of Dalmer and Moll [1933]. The *d*-ascorbic acid was tested on the scorbutic guinea-pig and found to be completely inactive in doses 40 times as high (20 mg. daily) as were required for cure with *l*-ascorbic acid. The other homologues tested were also inactive in doses of 4–20 mg. daily.

Summary.

1. Large doses of *l*-ascorbic acid (up to 5 g./kg. in one dose or up to 12 g./kg. total amount in the chronic test) were tolerated without symptoms by our animals. Histological examination of the organs showed no change in the kidney, liver, heart and lung. There was no evidence of hypervitaminosis C.

2. After administration of 0·1 g./kg. ascorbic acid subcutaneously to a dog 75 % was excreted in the urine during the next 9 hours.

3. The urine of animals receiving large doses of ascorbic acid is capable of curing scorbutic guinea-pigs in daily doses of 0·2–1 ml. daily.

4. Synthetic *l*-ascorbic acid behaved biologically identically with *l*-ascorbic acid from paprica.

5. *d*-Ascorbic acid is inactive in doses of 20 mg. daily. Of the various isomerides and homologues examined only *d*-erythro-3-ketohexonic acid showed a definite antiscorbutic action which was about 1/20 of that of *l*-ascorbic acid.

REFERENCES.

Dalmer and Moll (1933). *Z. physiol. Chem.* **242**, 116.
van Eekelen, Emmerie, Josephy and Wolff (1933). *Acta Brev. Néerl. Physiol. Pharm.* **3** (10/11), 168.
Harris, Ray and Ward (1933). *Biochem. J.* **27**, 2011.
Reichstein and Grüssner (1934). *Helv. Chim. Acta*, **17**, 311.
—— —— and Oppenauer (1933, 1). *Nature*, **132**, 280.
—— —— —— (1933, 2). *Helv. Chim. Acta*, **16**, 561, 1019.

CVIII. THE EFFECT OF IODOACETIC ACID AND OTHER SUBSTANCES ON THE DISAPPEARANCE OF GLUCOSE FROM SHED BLOOD.

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(*Received March 31st, 1934.*)

Recent work on the breakdown of glucose to lactic acid, while it has produced a number of new facts and hypotheses, has not by any means succeeded in producing a clear and unequivocal account of the reactions involved. Thus on the one hand we have the demonstration that iodoacetic acid, which prevents lactic acid formation in muscle, reacts with and destroys glutathione the co-enzyme of glyoxalase. On the other hand, it has been claimed that muscle, from which gluthathione has been removed by dialysis, is still capable of lactic acid production provided that adenosinetriphosphate and magnesium ions are present.

The experiments described in this paper were undertaken in the hope that shed blood, in which the process of glycolysis is continuous, would prove a simpler system than muscle, the underlying assumption being that in both tissues lactic acid formation followed the same course. As will be seen, this assumption proved not to be justified, but this fact in itself seems to be one of considerable importance.

Experimental methods.

Human venous blood was used, with addition of sodium oxalate as an anticoagulant. In some cases the blood was diluted with an equal amount of water (a procedure shown to be without appreciable influence on the rate of disappearance of sugar) and in some the p_H was adjusted to 8·5 by addition of sodium hydroxide solution. This adjustment of p_H, while it increased the rate of glycolysis in the control samples, as compared with blood at the normal p_H, did not affect the inhibition of glycolysis by iodoacetic acid *etc.* Measured amounts of this blood mixture were transferred to separate test-tubes and to them the various substances to be studied were added in $N/10$ solution. Acids were used in the form of sodium salts (at p_H 7·5) and $N/10$ NaCl was used as a control, and, where necessary, for adjustment of volume. Incubation was at 37°. Sugar estimations were made by the method of Hagedorn and Jensen.

Iodoacetic acid was prepared by the action of potassium iodide on chloroacetic acid and purified by repeated precipitation from benzene solution with light petroleum. Iodoethyl alcohol was synthesised by the method of Hendry [1889], the final product being decolorised by sodium thiosulphate and distilled *in vacuo*. *l*-Bromopropionic acid was synthesised by the method of Volhard [1887] and from it a specimen of *l*-iodopropionic acid was prepared by the method of Abderhalden and Guggenheim [1908]. The β-iodopropionic acid was a Kahlbaum preparation, kindly presented to us by Dr H. S. Rule.

[1] In receipt of a part-time grant from the Medical Research Council.

Results and discussion.

Iodoacetic acid proved to be the most efficient of the substances tested in preventing the disappearance of glucose. Nevertheless it was without appreciable effect in concentrations of the order capable, in cardiac muscle, of completely inhibiting anaerobic carbohydrate breakdown. Bromoacetic acid was almost as active, chloroacetic acid less so; whilst iodoethyl alcohol and α-iodopropionic acid displayed smaller though very definite powers of inhibition of glycolysis. β-Iodopropionic acid however was quite inactive (Table I).

In a number of experiments it was noticeable that iodoacetic acid, while completely inhibiting glycolysis from the end of the first hour of incubation to the seventh hour, allowed some apparent decrease of sugar during the first hour. In part this may be due to the time necessary for the iodoacetic acid to exert its effect—*i.e.* to react with glutathione. Since, however, the method of protein precipitation used in the Hagedorn and Jensen method does not completely remove non-carbohydrate reducing substances, it seems likely that this apparent decrease in the blood-sugar may be due to the destruction by the iodoacetic acid of glutathione and possibly other substances such as ergothioneine.

The experiment quoted in Table I (*a*) shows the inhibition well maintained at 24 hours. This was not always the case, though usually the control, even then, showed the lowest sugar content. Most probably this was due to the fact that no special precautions were taken to exclude bacterial contamination.

Table I. *Effect of iodoacetic acid, iodoethyl alcohol, α-iodopropionic acid, bromoacetic acid and chloroacetic acid on the rate of disappearance of sugar from blood.*

(*a*) 0·7 ml. *N*/10 NaOH added to each 2·0 ml. blood. 0·25 ml. *N*/10 solution of reagent (neutral) or NaCl added per 5·0 ml. of blood mixture. Incubation at 37°.

mg. sugar per 100 ml. blood.

Time hours	Control	Iodoacetic acid	Iodoethyl alcohol	α-Iodopropionic acid
0	87	87	87	87
1	79	84	82	77
3	55	84	81	69
5	55	84	75	59
7	37	85	61	57
24	35	80	59	57

(*b*) As above, but 0·4 ml. *N*/10 chloroacetic acid, 0·2 ml. *N*/10 bromoacetic acid or *N*/10 iodoacetic acid (neutralised) and volumes adjusted with *N*/10 NaCl.

mg. sugar per 100 ml. blood.

Time hours	Control	Iodoacetic acid	Bromoacetic acid	Chloroacetic acid
0	225	225	225	225
3	201	224	215	209
5	179	209	211	91

Blood from a case of diabetes.

Chloro-, bromo- and iodo-acetic acids are all known [Dickens, 1933] to react with glutathione *in vitro*, and the velocity constants of the reactions have been determined. The action between glutathione and chloroacetic acid proceeds very much more slowly than those between glutathione and bromo- and iodo-acetic acid. In accordance with this, chloroacetic acid is much less efficient than the

other two substances in preventing glycolysis, but it is rather surprising to find it as efficient as it is—roughly half as efficient as bromoacetic acid. This is particularly so when one remembers that even in relatively greater concentrations it is without inhibitory action on the lactic acid production of cardiac muscle [Gaddie and Stewart, 1934]. These facts immediately suggest that the mechanism of sugar breakdown in blood differs from that in muscle and that the action of chloroacetic acid is not confined to the destruction of glutathione.

It is obviously desirable to test the effect of glutathione on the other two substances capable of inhibiting glycolysis (iodoethyl alcohol and α-iodopropionic acid) as well as the related compound, β-iodopropionic acid, which had no such effect. This became especially important when it was shown [Gaddie and Stewart, 1934] that none of these substances had an inhibitory effect on the frog's heart comparable with that exerted by iodo- and bromo-acetic acids. The method of testing was as follows: Solutions of the substance to be tested ($M/10$) and glutathione ($M/100$) were prepared at p_H 7·5, and 10 ml. of each solution were mixed with 10 ml. of a phosphate buffer at the same p_H. From this mixture, 5 ml. were at once removed and examined immediately for hydriodic acid. Further samples were removed at intervals of 15 minutes (the mixture meanwhile standing at room temperature) and tested in the same way. Control experiments were made without glutathione. Estimation of hydriodic acid was made by adding to the sample 2 ml. of $N/10$ potassium ferricyanide containing zinc sulphate, and 2 ml. of 3 % acetic acid, extracting the liberated iodine with chloroform (three extractions using 2 ml. of chloroform each time) and titrating the chloroform extracts with $N/200$ sodium thiosulphate. This method was tested on standard solutions of potassium iodide and was found to give satisfactory results.

When β-iodopropionic acid was tested in this way, the reason of its inactivity in preventing glycolysis became apparent at once—even in the absence of glutathione it lost hydriodic acid with much greater rapidity than did iodoacetic acid in presence of glutathione.

The α-iodopropionic acid, however, was stable alone and reacted with glutathione much more slowly than did iodoacetic acid (Table II), a finding in keeping with its smaller power of preventing the disappearance of sugar.

Table II. *Effect of iodoacetic acid, iodoethyl alcohol and α-iodopropionic acid on glutathione* in vitro.

ml. $N/100$ thiosulphate used in titrating iodine liberated per 5 ml. of solution.

Time min.	Iodoacetic acid	Iodoethyl alcohol	α-Iodopropionic acid
0	0·540	0·03	0·170
20	1·055	0·02	0·425
40	1·100	0·02	0·575
60	1·150	0·025	0·660

Iodoethyl alcohol, on the other hand, failed to react with glutathione, or rather, failed to liberate hydriodic acid in the presence of glutathione, although it was perfectly capable of inhibiting glucose breakdown. This can only mean that iodoethyl alcohol reacts with glutathione in some other way, or that its effect is exercised entirely on some other part of the system responsible for the conversion of glucose into lactic acid. The first of these alternatives seems to be excluded by the finding that the sulphydryl group (probably the essential part of the molecule for co-enzyme activity, and the most likely part to undergo

change) is unaffected by incubation with iodoethyl alcohol at p_H 7·5. This is shown by the fact that after incubation of the mixture for 1½ hours the amount of sulphydryl present is undiminished when tested both colorimetrically with sodium nitroprusside and titrimetrically with iodine. Still further evidence of the independence of glutathione and iodoethyl alcohol is afforded by the fact that added glutathione does not abolish the inhibitory action of iodoethyl alcohol on glycolysis, whereas it does abolish the action of iodoacetic acid (Table III).

Table III. *Effect of added glutathione on the inhibition of glycolysis in presence of iodoacetic acid and iodoethyl alcohol. Experiment as in Table I but 0·5 ml. reagent per 5 ml. blood mixture, and 20 mg. glutathione (in neutral solution) volumes adjusted with N/10 NaCl.*

mg. sugar per 100 ml. blood.

Time hours	Control	Iodoacetic acid	Iodoacetic acid and glutathione	Iodoethyl alcohol	Iodoethyl alcohol and glutathione
0	103	103	105	104	103
1	91	93	95	90	91
3	55	93	56	83	85
5	49	91	54	75	74

We are therefore driven to the conclusion that the actions of iodoacetic acid and iodoethyl alcohol, though leading to the same end-result, are fundamentally different at least in part. Moreover, since iodoethyl alcohol inhibits glycolysis in blood with an activity roughly half that of iodoacetic acid, but is totally ineffective in cardiac muscle at a concentration tenfold that of iodoacetic acid capable of complete inhibition of glycolysis, it follows that some part of the glycolytic mechanism present and of importance in blood is absent from cardiac muscle, or at least is not of critical importance in the latter.

The only alternative to this hypothesis appears to be the idea that iodoethyl alcohol is acting merely as a general enzyme poison. Indeed, when one considers the quantity involved, such an idea might be entertained with regard not only to iodoethyl alcohol, but also with regard to iodoacetic acid itself. For there is no proof that the whole action of this latter substance consists in its destruction of the co-enzyme of glyoxalase, and one might consider the restorative effect of added glutathione (which seems to be complete) as being merely an *in vitro* reaction resulting in the destruction of the enzyme poison and the replacement of the co-enzyme previously lost.

We have not tested this alternative hypothesis completely, and, indeed, there are not many enzyme systems in blood which have been thoroughly studied. We have, however, tested the effect of both iodoacetic acid and iodoethyl alcohol on the blood-phosphatase. The blood, made alkaline to p_H about 8·5 by means of sodium hydroxide was incubated with excess of sodium glycerophosphate solution and addition of sodium iodoacetate or iodoethyl alcohol in the concentrations known to inhibit glycolysis. In both cases samples withdrawn at 24 and 48 hours showed decreased hydrolysis of the organic phosphate as compared with the controls (Table IV). Since however the phosphatase may form part of the system responsible for the breakdown of glucose to lactic acid, this cannot be taken as evidence that the two iodo-compounds act as general enzyme poisons. It is perhaps noteworthy that they are without any inhibitory effect on the xanthine oxidase of milk.

Table IV. *Effect of iodoacetic acid and iodoethyl alcohol on blood-phosphatase.*

mg. P per 100 ml. blood.

Time (hours)	0	24	48
Control	7·44	27·18	39·17
Iodoacetic acid	7·18	21·44	31·63
Control	6·40	38·30	47·11
Iodoethyl alcohol	6·23	28·30	30·45

SUMMARY.

Iodoacetic acid inhibits the disappearance of glucose from shed blood, but only in concentrations much higher than those required to prevent lactic acid formation in cardiac muscle. Bromoacetic acid has an almost equal effect. Chloroacetic acid, α-iodopropionic acid and iodoethyl alcohol have similar, though weaker actions. β-Iodopropionic acid is without effect probably on account of the rapidity with which it loses iodine.

The effect of iodoacetic acid, but not that of iodoethyl alcohol, is counteracted by addition of glutathione. Iodoethyl alcohol is also peculiar in not reacting with glutathione *in vitro* to produce hydriodic acid. Both iodoacetic acid and iodoethyl alcohol, in concentrations capable of preventing sugar disappearance, inhibit the action of blood-phosphatase.

The significance of these findings is discussed in relation to the mechanism of carbohydrate breakdown.

REFERENCES.

Abderhalden and Guggenheim (1908). *Ber. deutsch. chem. Ges.* **24**, 2855.
Dickens (1933). *Biochem. J.* **27**, 1141.
Gaddie and Stewart (1934). *J. Physiol.* **80**, 457.
Hendry (1889). *Bull. Acad. Roy Belgique* (3), 182.
Volhard (1887). *Liebig's Ann.* **242**, 161.

CIX. A NOTE ON THE GENERAL INTERPRETATION OF FATTY ACID ANALYSES BY THE ESTER FRACTIONATION METHOD.

By THOMAS PERCY HILDITCH.

From the Department of Industrial Chemistry, University of Liverpool.

(*Received April 12th, 1934.*)

MISCONCEPTIONS apparently persist, in the minds of some chemists, as to the degree of exactitude obtainable in, or claimed for, the analytical procedure which we, in common with a number of other investigators, have now employed for some years. It is desired, therefore, to make a brief general statement on the matter. Let it be clearly pointed out in the first place, that there are at least two essentially different general problems with which the student of the mixtures of fatty acids combined in natural fats is faced:

(*a*) Knowledge of the relative proportions, as well as of the individual acids, of what may be termed the *major component acids* of different natural fats. A "major component acid" is defined, broadly speaking, as one which occurs in quantities of from, say, 5–10 % upwards of the mixed fatty acids of a fat (and which very often amounts to 30, 40, 50 % or even more, of the total acids);

(*b*) Isolation and characterisation of acids which are sometimes present in much smaller proportions, and which form, for example, less than 1 % of the whole of the mixed fatty acids.

The essential difficulty of quantitative analysis of natural fatty acid mixtures resides in the presence therein of nearly consecutive members, saturated and unsaturated, of a single homologous series of compounds—acids, that is, of the C_{12}, C_{14}, C_{16}, C_{18}, C_{20}, C_{22}, *etc.* series. At the same time, natural fatty acid mixtures differ widely in complexity; for example:

	Major component fatty acids	
	Saturated	Unsaturated
Seed-fats of the cacao butter, *etc.*, type	C_{16}, C_{18}	C_{18}
Palm oil, tallow, lard, many vegetable oils	(C_{14}), C_{16}, C_{18}	C_{18}
Seed-fats of the *Palmae*	C_8, C_{10}, C_{12}, C_{14}, C_{16}	C_{18}
Milk-fats	C_4, C_6, (C_8, C_{10}, C_{12}), C_{14}, C_{16}, C_{18}	C_{18}
Marine animal fats	(C_{14}), C_{16}, (C_{18})	(C_{14}), C_{16}, C_{18}, C_{20}, C_{22}, (C_{24})

The ester-fractionation procedure is not suitable as a rule for quantitative determination of minute proportions of fatty acids (class (*b*) above), since an accuracy to within fractions of a unit % (of the total mixed fatty acids) cannot in general be reached by the method. On the other hand, for studies of the relative proportions of the major component acids of natural fats (class (*a*) above)—an aspect of fat-chemistry with which the writer and his colleagues have been engaged for a somewhat long period—the method gives results which,

at least with many of the simpler kinds of fats illustrated above, are reliable to about one unit %. Such an order of accuracy, or even somewhat less, is quite sufficient to have demonstrated, for example, many cases of empirical but close relationship between fatty acid compositions and biological origin; or, again, in one category of animal depot fats, to have established the relative constancy of palmitic acid content and the approximate stearic-oleic acid balance, which we have shown to be characteristic for fats of varying average unsaturation in this group.

Component fatty acids are given, therefore, to the first decimal place (in conformity with the usual convention that one more significant figure is given than that upon which reliance may be placed); also, the data are expressed as the percentage proportions of the *mixed fatty acids* estimated (so that the circumstance that the totals amount to exactly 100·0 in all cases has no bearing upon the experimental accuracy or otherwise of the determination). Moreover, it sometimes happens that the calculation ultimately results in the appearance of a fractional percentage (*e.g.* 0·7 % myristic acid, Table I, p. 781) of an acid present in small amount; in such cases of course this does not imply that narrower limits of accuracy are claimed for the analytical procedure employed than those already stated. These explanatory remarks will probably be superfluous to most workers in this field, but apparently the points referred to have not always been appreciated by some of our fellow-investigators.

When the ester-fractionation process is examined critically, it is at once evident that its order of accuracy depends almost wholly on the answer to the question: Is it possible to obtain ultimate ester-fractions in any one of which no more than two adjacent homologous saturated, and two adjacent homologous unsaturated, members are present? In all probability, absolutely complete separation in this respect is rarely, if ever, attainable; but due observance of two precautions adequately safeguards the analysis and leads ultimately to the presence of not more than traces of a third homologue in the fractions finally obtained:

(*a*) In the first place, separation by fractional distillation of esters is never attempted before having recourse to as thorough a separation as possible of saturated from unsaturated acids by the lead salt-alcohol process. This removes all stearic and higher saturated acids and very nearly all palmitic acid from the unsaturated acids. In the common cases therefore where mixtures of palmitic, stearic, oleic and linoleic acids (with perhaps minor amounts of myristic or other acids) are concerned, this simplifies very much the character of the two groups of esters which have to be distilled. In other and more complex instances, such as animal milk fats, which contain a good deal of saturated acids of lower molecular weight than palmitic, or the marine animal oils, with a mixture of homologous unsaturated acids, the lead salt separation is even more vital a part of the process; for here, even when it does not result in an almost complete division as between saturated and unsaturated acids, it still has the effect of removing almost all the palmitic and all higher saturated acids from the so-called "liquid" acids, with the consequence that there is a much greater differentiation in the relative molecular weights (and therefore boiling-points of the esters) of the saturated and unsaturated acids present in the "liquid" esters than exists in the original mixed acids as a whole, in which the C_{16} and higher saturated acids are also present.

(*b*) In the second place, in addition to such refractionation of primary fractions as may be necessary, it is our practice to collect a large number of fairly small final ester fractions, whether the composition of the mixed esters is

relatively simple or not. This multiplication of fractions minimises any error due to the possible retention of traces of a third homologue in fractions consisting almost wholly of two adjacent homologues, and also tends to reduce the effect of small positive and negative errors in the numerous equivalent and iodine value determinations involved.

Comparison of the closely similar compositions deduced by use of this method, in this laboratory and elsewhere, for the acids in different specimens of the same kind of fat is sufficient evidence of the fact that at all events it leads to results which are very concordant; but it may be argued that this does not necessarily imply their accuracy. On the other hand, the mixture of acids from a completely or partially hydrogenated fatty oil is of an essentially different type, so far as the analysis is concerned, from that from the original fatty oil, since the proportion of saturated acids is entirely altered by hydrogenation. A comparison of the results obtained from analyses of original and hydrogenated fats thus affords a somewhat rigorous test of the general accuracy of the procedure. A few instances of the kind, drawn from data in our laboratory records, will therefore be given by way of illustration; it is of course necessary to compare the molar percentages of the various homologous acids present in each case.

Table I.

Palm oil fatty acids (mol. %).

	Original		After hydrogenation	
Myristic	1·9		0·7	
Palmitic	34·3		35·5	
Stearic	5·3	63·8	62·7	63·8
Oleic	50·6		1·1	
Linoleic	7·9		—	

Pig back fat fatty acids (mol. %).

	Original		Partially-hydrogenated (1)		Partially-hydrogenated (2)	
Myristic	3·5		2·8		2·2	
Palmitic	26·6		25·6		28·1	
Stearic	14·0	69·0	18·1	69·9	28·2	68·7
Oleic	41·4		50·9		40·5	
Linoleic	13·6		0·9		—	
C_{20-22} unsaturated	0·9		1·7		1·0	

Elasmobranch liver oil fatty acids (mol. %).

		Original		After hydrogenation
C_{14}	Saturated	1·4	1·6	2·5
	Unsaturated	0·2		—
C_{16}	Saturated	15·3	19·4	17·5
	Unsaturated	4·1		—
C_{18}	Saturated	1·4	38·6	39·8
	Unsaturated	37·2		—
C_{20}	Saturated	1·1	16·8	16·8
	Unsaturated	15·7		—
C_{22}	Saturated	—	13·9	13·4
	Unsaturated	13·9		—
C_{24}	Saturated	—	9·7	10·0
	Unsaturated	9·7		—

It seems therefore that an accuracy of about one unit % can be relied upon for the fatty acids of the simpler fats while, as suggested by Guha *et al.* [1930], in

the more complex fats, especially in the marine animal oils, the figures may be less reliable and the order of accuracy perhaps only within 3–4 units %. Even the latter represents a not unreasonable standard of accuracy, compared with that attainable in the quantitative assay of many other natural products of a complexity comparable with that of most fat mixtures. Moreover, it has sufficed to demonstrate general relationships amongst the natural fats of the kinds to which reference has already been made; while the detection of palmitoleic acid in bird and rat fats (discussed in recent papers) is entirely due to the use of the ester-fractionation method.

Some recent work on the linoleic acid content of butter-fat by Bosworth and Brown [1933] and by Eckstein [1933] will now be considered. Bosworth and Brown succeeded, as a result of repeated fractionation of the mixed esters from over 11 kg. of butter-fat, in isolating and defining the minute proportions of decenoic and tetradecenoic acids which (as Grün *et al.* [1922; 1924] had previously shown) occur in butter. This outstanding contribution to knowledge of the minor component fatty acids of butter suggests that decenoic acid may form 0·18 % of the total fatty acids and, since the authors actually isolated an amount of tetradecenoic acid smaller than that of decenoic acid, it seems fair to assume that tetradecenoic acid occurs in still smaller quantities. (Bosworth and Brown consider however that the original butter must have contained 0·87 % of tetradecenoic acid.) They also show that highly unsaturated acids (probably of the C_{22} series) are present in butter-fat; and in an earlier paper [Brown and Sutton, 1931] the presence of such acids to the extent of 0·3 % was demonstrated.

In the course of their work however the authors state that they have been unable to demonstrate the presence of ordinary linoleic acid and suggest that the unsaturation higher than that of oleic ester, which invariably has been observed in our "liquid" ester fractions corresponding in equivalent to those of C_{18} unsaturated acids, is due to imperfect fractionation and contamination of these fractions by either the mono-ethenoid C_{10} and C_{14} or the polyethenoid C_{22} esters, or both.

Unfortunately, Bosworth and Brown, although they refer to our use of the methyl ester distillation method, have based their fractionation practice on the earlier work of Crowther and Hynd [1917], Holland and Buckley [1918–23], and Channon *et al.* [1924]. They prepared their esters by direct "methanolysis" of butter-fat and employed no preliminary resolution of the mixed acids into predominantly saturated and predominantly unsaturated groups. Moreover, they refer to criticisms advanced by Channon *et al.* against the ester-fractionation method, but overlook the very complete refutation of these criticisms which followed almost immediately from Armstrong *et al.* [1925]—a refutation which, so far as the present author is aware, has been accepted. (For example, recently, Drummond and Gregory [1932] refer to certain analyses as having been made "by the more accurate fractionation method.")

The iodine values of the acids in the higher methyl ester fractions obtained by Bosworth and Brown do not, it is true, suggest the presence of C_{18} unsaturated acids less saturated than oleic. This is natural, for, owing to the absence of any lead salt separation, methyl stearate and also (judging from the recorded mean molecular weights) fairly large amounts of methyl palmitate are present in all the fractions concerned. Since, in our experience, the minimum amount of stearic acid which has been observed in any butter-fat is greater than the maximum amount of linoleic acid which we have ever suggested to be present, it follows that even if complete separation of palmitic and C_{18} esters had been

achieved, the iodine value of their C_{18} esters would still have been somewhat below that of methyl oleate. Bosworth and Brown however applied a lead salt-ether separation to the acids from their higher ester fractions and supplemented this by attempted resolution of the unsaturated acids by means of the barium salts in benzene, and they conclude that practically all polyethenoid acids present are of higher molecular weight than the C_{18} series. The absence of data as regards the relative weights of the various fractions obtained in this part of their work limits any discussion to a qualitative basis, but it must be pointed out that the mean molecular weights of the acids from the insoluble (as well as from the soluble) barium salts are, in six instances out of nine, considerably higher than those of the original mixtures.

Bosworth and Brown, from an examination of the ether-insoluble bromo-addition products, were unable to demonstrate the presence of ordinary linoleic acid, or of more highly unsaturated acids of the C_{18} series. Eckstein [1933], employing the same method, finds evidence of only traces (of the order of 0·1–0·3 %) of linoleic and linolenic acids. The latter author also shows that addition of 5 % of the esters of either of these acids (prepared by the method of Rollett [1909]) to butter results in the mixed fat showing bromine values corresponding to 50 % or less of the added linoleic or linolenic acid; he obtained however by fractionation of the "liquid" methyl esters of a Detroit butter iodine values of 93, 98 and 117 for the three highest-boiling fractions.

Now, in all our analyses of butter-fat "liquid" methyl esters, there have been obtained consistently large fractions of esters with equivalents approximating to those of the C_{18} group, the iodine values of which range from about 92 to 99, that is, from 8–15 % above that of methyl oleate. In each of our papers on butter-fats [1929; 1930; 1933] only one instance was given, in each case, of the full details of the fractionation data, in an endeavour to save space. Seven other instances, taken from the material dealt with in these previous papers, will now be cited in order to emphasise the invariable nature of these observations. In doing so (again for considerations of space) only the weights, saponification equivalents and iodine values of the three or four highest-boiling fractions of the "liquid" esters will be quoted in each case, together with the total weights of "liquid" esters distilled and the proportion of the total fatty acids represented by the "liquid" fatty acids; the latter data are very material to the present argument.

Inspection of the figures (Table II) shows that the ester fractions of iodine value 92–99 to which attention is invited represent from 15 to over 20 % of the total fatty acids of the butter-fats. Bosworth and Brown [1933] say "This high iodine number can easily be explained now by the presence of low molecular weight acids with one double bond and iodine numbers greater than that of oleic acid together with appreciable amounts of unsaturated acids of high molecular weight with two or more double bonds." This statement is untenable for the following reasons. The iodine value of decenoic acid is 149·3 and of tetradecenoic acid 107·7. It would therefore require a mixture of about 50 % each of methyl tetradecenoate and oleate, or of about 20 % methyl decenoate with 80 % methyl oleate to give iodine values in the neighbourhood of 95; the percentage of a tetra-ethenoid C_{22} ester (iodine value 294) required to raise the iodine value of methyl oleate and the latter to a mean value of 95–97 would be less, namely, about 5 %. Consequently, the amount of any or all of these esters necessary to be present in our C_{18} ester fractions in order to produce the observed results would have been many times greater than the total amounts of these acids present in the whole of the butter-fat, according to Bosworth and Brown.

Table II.

Publication	Butter-fat No.	Proportion of "liquid" acids from total butter-acids (%)	Weight of "liquid" esters distilled g.	"Liquid" methyl ester fractions			
				No.	g.	Sap. equiv.	I.V.
Hilditch and Jones [1929]	"B"	30·7	91·41	L 3	19·07	290·1	91·0
				L 4	8·47	293·0	94·9
				L 5	26·29	293·6	99·3
				L 6*	7·17	325·2	103·0
Hilditch and Sleightholme [1930]	"I"	47·5	69·43	L 4	9·55	292·9	92·1
				L 5	7·33	294·5	93·5
				L 6	7·83	295·8	94·1
				L 7*	8·45	307·1	105·0
"	"V"	41·8	105·15	L 4	13·10	290·1	91·7
				L 5	9·27	293·5	95·1
				L 6	15·59	295·2	98·9
				L 7*	7·12	327·7	118·6
Bhattacharya and Hilditch [1931]	Cow ghee no. 2	44·2	48·70	L 4	8·63	293·8	92·4
				L 5	9·15	294·4	96·8
				L 6*	5·32	303·8	103·3
Dean and Hilditch [1933]	"Winter ration"	48·5	120·24	L 4	16·46	290·3	92·8
				L 5	20·75	293·5	94·0
				L 6	10·47	296·1	96·2
				L 7*	7·40	324·0	95·3
"	"Intermediate period"	52·0	120·31	L 3	14·35	291·4	90·8
				L 4	15·64	293·1	94·4
				L 5	16·52	294·2	96·3
				L 6	10·93	294·8	97·9
				L 7*	15·97	308·1	101·5
"	"Summer pasture"	52·2	120·61	L 4	15·63	292·4	91·6
				L 5	14·17	294·2	93·4
				L 6	20·25	295·0	96·4
				L 7*	15·31	308·3	99·3

* The final fractions, in each case, are the residual esters which remained undistilled; the iodine values of these are always notably higher than those of the preceding distillate, and doubtless this is due to the small traces of C_{22} unsaturated acids observed by Brown *et al.*, as well as to unsaponifiable matter (*e.g.* the residual esters, I.V. 118·6, from fat "V" above, still possessed an I.V. of 103·6 and an equivalent of 303 after removal of unsaponifiable matter). We have never attempted to allow, in the butter-fat analyses, for anything but C_{18} unsaturated esters and unsaponifiable in the residual fractions, because the iodine values thereof are probably of doubtful accuracy owing to the possibility of some thermal decomposition; we have therefore applied, for purposes of calculation, the iodine value of the preceding fraction to the C_{18} esters in the residues.

(In the instance referred to, allowance for C_{22} esters would lead to the fraction L 7 being recorded as 5·80 g. C_{18} and 0·78 g. C_{22} esters with 0·54 g. unsaponifiable, whereas our calculation (with C_{18} esters alone) was 6·43 g. C_{18} esters and 0·69 g. unsaponifiable. On the whole fat, this would lead to about 0·2 % diminution in the recorded C_{18} unsaturated acids and correspondingly to 0·2 % of C_{22} acids, calculated on this basis.)

The writer remains convinced, then, that these characteristic iodine values in the neighbourhood of 95 are due to unsaturated C_{18} esters. Bosworth and Brown state that there is no tetra-ethenoid C_{18} ester present, and Eckstein fails to find more than about 0·3 % at most of linoleic and linolenic esters in the butter-fat as a whole. Hilditch and Jones [1929] also state that they failed to isolate either any tetrahydroxystearic acid or any definite brominated acid (other than a trace insoluble in ether which darkened without definitely melting at 170–180°) from the ester fractions of iodine value 97–98. The consistent presence of C_{18} ester fractions corresponding to 15–20 % of the total butter-fatty acids with an average unsaturation more than 10 % in excess of that of methyl oleate nevertheless still requires explanation.

It is hoped to return to the investigation of this question in due course, but it will necessarily be some considerable time before any further report can be made on such experiments, and for this reason it has been thought best to point out the above facts in the present communication. It need only be added that Eckstein's experiments with regenerated linoleic and linolenic acids (presumably originating from seed fats) merely indicate that the ordinary linoleic acid of seed fats is not present to any marked extent in butter, and that Smith and Chibnall [1932] had similar difficulty in correlating the brominated products from the glyceride fatty acids of forage grasses with those from the ordinary linoleic acid of seed fats. Possibly this may afford a clue to the solution of an admittedly puzzling problem. In the meantime, the quantitative data, to which attention has here been redirected, must be taken into consideration in computing the general composition of butter-fatty acids, and the writer has adopted the view, from the 1929 paper onwards, that, in the absence of definite characterisation, the general distribution of C_{18} unsaturated acids in nature favours the assumption that the most likely polyethenoid C_{18} unsaturated acid to be present is diethenoid rather than still less saturated in character.

REFERENCES.

Armstrong, Allan and Moore (1925). *J. Soc. Chem. Ind.* **44**, 63T.
Bhattacharya and Hilditch (1931). *Analyst*, **56**, 161.
Bosworth and Brown (1933). *J. Biol. Chem.* **103**, 115.
Brown and Sutton (1931). *J. Dairy Sci.* **14**, 134.
Channon, Drummond and Golding (1924). *Analyst*, **49**, 311.
Crowther and Hynd (1917). *Biochem. J.* **11**, 139.
Dean and Hilditch (1933). *Biochem. J.* **27**, 889.
Drummond and Gregory (1932). *Z. Vitaminforsch.* **1**, 257.
Eckstein (1933). *J. Biol. Chem.* **103**, 135.
Grün and Winkler (1924). *Z. angew. Chem.* **37**, 228.
—— and Wirth (1922). *Ber. deutsch. chem. Ges.* **55**, 2197.
Guha, Hilditch and Lovern (1930). *Biochem. J.* **24**, 266.
Hilditch and Jones (1929). *Analyst*, **54**, 75.
—— and Sleightholme (1930). *Biochem. J.* **24**, 1098.
Holland and Buckley (1918–23). *J. Agric. Res.* **12**, 719; **24**, 365.
Rollett (1909). *Z. physiol. Chem.* **62**, 410.
Smith and Chibnall (1932). *Biochem. J.* **26**, 218.

CX. THE BODY FATS OF THE HEN.

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THE examination of bird body fats has hitherto been restricted to separation of the saturated acids present by means of their lead salts (Twitchell) or by oxidation (Bertram). Bömer and Merten [1922] state that the acids present in a sample of goose fat were palmitic (21·8 %), stearic (3·9 %) and oleic (74·3 %), while Grossfeld [1930; 1931] reports for the same fat lower volatile acids (0·2 %), palmitic (20·9 %), stearic (10·6 %), oleic (49·0 %) and linoleic (19·3 %) acids[1]. The latter author also gives two similar component fatty acid analyses for chicken fat as follows: lower volatile acids (0·1 %), palmitic (18·4, 19·3 %), stearic (8·9, 7·5 %), oleic (54·7, 55·4 %) and linoleic (17·9, 17·8 %) acids. Grossfeld pointed out however that the percentage of palmitic acid, calculated from the saponification value of the total mixed fatty acids, was about 10 units higher than that calculated from the molecular weight of the separated "solid" fatty acids and attributed this to the presence of fatty acids of lower molecular weight than that of palmitic acid.

The work about to be recorded proves that this discrepancy is not due in hen body fats to the presence of lower saturated fatty acids, but to that of about 7–8 % of a palmitoleic acid. Indications of this or a similar acid were recently reported by Banks *et al.* [1933] in rat body fats, although lack of material prevented decisive evidence being obtained.

The primary objectives of our experiments were to ascertain the general nature of a bird fat and to compare the depot fats from different parts of the body. We were fortunate in having the co-operation of Dr E. M. Cruickshank, who not only supervised the rearing of the fowls, but was also good enough to separate for us the fatty tissues from the abdominal layers, gizzards[2] and necks of the birds. We wish to express our great appreciation of the help thus afforded to us by Mr Halnan and Dr Cruickshank of the Cambridge School of Agriculture.

The specimens of the three body fats which we examined in the first instance were from 12 Light Sussex hens, 2 years old, whose diet had included 7 % of fish meal containing about 2·5 % of fish fat. The results of analyses by the ester fractionation method showed, as stated, that palmitoleic acid and also small amounts of unsaturated acids of the C_{20} and C_{22} series were present; and a new series of fats from 24 Light Sussex hens (which had received no fish meal), 7 months old, was therefore studied, in order to ascertain whether these acids

[1] The figures given here are the percentages of each acid on the *mixed component fatty acids*, and not (as in the original memoirs) those in the whole fats.

[2] The "gizzard fat" consisted of the layer of fat adherent to the gizzard together with the fat of the mesenteric membrane and any which was packed around the kidneys. Roughly speaking therefore this fat might perhaps be compared with the leaf and inner back fats of the pig, whilst the abdominal layer is in some respects more akin to the outer layers of back fat of the pig.

originated from those present in the fish meal fat. The results however were closely similar to those for the original group, and it is inferred that these acids are normal components of hen depot fats.

Since the diet of the second batch of 24 hens did not contain fish meal, it is perhaps preferable to deal with them first in the present paper. The diet of each group of birds, from 6 weeks after hatching onwards[1], was made up of mash (bran, maize and Sussex ground oats 20 parts each and sharps 40 parts) and grain (maize and wheat). For the group of 24 hens, 10 % of extracted soya bean meal and 2 % of mineral mixture were added to the mash, whilst to the mash given to the group of 12 hens there were added 7 % each of extracted soya bean meal and fish meal (containing 2·4 % fat) and 3 % of mineral mixture.

The average analyses of the rations were as follows:

	Moisture %	Ether extract %	Protein %	Carbo-hydrate %	Fibre %	Ash %
24 hens (fish meal-free)	12·4	4·0	16·1	55·3	6·5	5·7
12 hens (with fish meal)	12·5	3·8	14·1	57·9	6·6	5·1

The fats were extracted from the tissues by boiling with acetone; at the ordinary temperature they consisted chiefly of deep-yellow liquids from which a certain quantity of white solid glycerides deposited. Their general characteristics are given in Table I.

Table I. *General characteristics of hen fats.*

	Yield of fat from adipose tissue %	Extracted fat			Mixed acids setting-point ° C.
		Sap. equiv.	I.V.	Acid value	
From 24 hens (fish meal-free diet):					
Abdominal	97·5	285·5	80·1	0·7	34·5
Gizzard	96·9	286·0	78·8	1·7	35·1
Neck	96·0	285·9	79·8	1·4	34·6
From 12 hens (7 % fish meal in diet):					
Abdominal	96·4	286·3	78·5	0·7	36·2
Gizzard	95·8	284·5	79·7	5·6	36·5
Neck	88·7	284·8	77·4	1·2	36·5

The component acids present in each fat were separated by means of lead salts into "solid" and "liquid" acids, after which the methyl esters of each of these groups of acids were fractionally distilled in a vacuum. Analyses of the esters of the "solid" acids furnished quite normal results, but those of the esters of the "liquid" acids showed two unusual features. In the first place, the penultimate and the residual ester fractions in this group possessed analytical characteristics which indicated the presence of small amounts of unsaturated acids of the C_{20} and C_{22} series; the proportions present were approximately calculated as described by Banks and Hilditch [1932], who encountered a similar instance in the case of sow body fats. In the second place, the saponification equivalents of the most volatile fractions of the "liquid" esters (obtained by refractionation of the lowest-boiling primary fraction) were unusually low, and when the unsaturated material present therein was calculated on the assumption

[1] During the first 6 weeks the birds were in confinement and their ration included minute amounts of olive oil containing calciferol (0·001 ml. per bird daily); after 6 weeks the birds had access to free range.

Table II. *Complete analytical data for the abdominal and gizzard fats from the group of 24 hens.*

(i) Lead salt separation of mixed fatty acids.

	Abdominal layer fat		Gizzard fat	
	g.	%	g.	%
"Solid" acids S	91·1	31·8	89·6	31·1
"Liquid" acids L	195·0	68·2	198·6	68·9

(ii) Fractional distillation of the methyl esters of the "solid" acids (S).

No.	g.	Sap. equiv.	I.V.	g.	Sap. equiv.	I.V.
S 1	4·16	268·9	1·4	3·59	269·5	1·3
S 2	4·71	269·9	1·4	5·87	269·8	1·4
S 3	7·93	270·8	1·4	8·02	270·4	1·6
S 4	11·77	272·3	1·8	9·75	272·0	1·8
S 5	10·86	273·5	2·3	15·53	272·5	2·0
S 6	11·62	274·9	3·0	14·76	276·1	3·2
S 7	11·89	278·9	4·2	14·63	280·9	4·8
S 8	10·37	288·5	6·6	6·01	289·2	6·6
S 9	3·46	297·6	10·8	6·22	296·9	9·5
	76·77			84·38		

(iii) Fractional distillation of the methyl esters of the "liquid" acids (L).

No.	g.	Sap. equiv.	I.V.	g.	Sap. equiv.	I.V.
L 1	29·53	286·1	104·8	33·92	284·4	104·1
L 2	11·59	291·6	112·8	9·94	291·7	116·5
L 3	12·13	293·1	114·0	10·86	292·9	117·4
L 4	10·99	295·1	115·4	9·89	293·6	117·6
L 5	11·47	295·4	116·0	11·94	293·6	119·6
L 6	12·18	296·5	114·5	6·87	294·8	118·1
L 7	4·65	312·7	114·8	7·23	308·6	123·2
	92·54	(297·8)*	(116·0)*	90·65	(297·1)*	(119·9)*

(iv) Determination of saturated esters in fractions L 1.

Wt. oxidised (g.)	25·79	30·15
Wt. of saturated esters recovered (g.)	2·24	2·92
I.V. of saturated esters recovered	1·0	3·9
Sap. equiv. of saturated esters recovered	267·7	269·9

(v) Fractional distillation of the hydrogenated methyl esters of the "liquid" acids (HL).

No.	g.	Sap. equiv.	I.V.	g.	Sap. equiv.	I.V.
HL 1	2·83	279·0	—	4·86	280·2	1·2
HL 2	3·02	283·1	—	9·65	287·5	1·5
HL 3	6·10	287·2	—	12·00	290·6	1·9
HL 4	9·23	290·7	—	13·40	293·9	2·0
HL 5	11·77	293·3	—	14·33	295·8	2·1
HL 6	11·47	295·9	—	14·89	297·0	2·1
HL 7	13·88	296·3	—	9·18	298·8	1·9
HL 8	13·66	297·6	—	7·58	313·6	10·1
HL 9	7·02	299·0	0·3	—	—	—
HL 10	5·42	306·9	1·8	—	—	—
	84·40	(301·2)*	(1·7)*	85·89	(299·9)*	(6·9)*

* Saponification equivalents and iodine values of residual esters freed from unsaponifiable material.

that only unsaturated esters of C_{18} acids were present, the consequent equivalent of the saturated esters indicated the presence of methyl laurate or of esters of even lower molecular weight [*cf.* Grossfeld, 1930; 1931]. Careful qualitative examination of the acids present in these most volatile fractions showed on the other hand that the saturated acid actually present was mainly palmitic, probably accompanied by minor amounts of myristic acid; and no trace could be detected of acids of lower molecular weight than myristic acid.

The methyl esters of the "liquid" fatty acids were therefore divided into two halves, one of which was fractionally distilled without further treatment, while the other was similarly treated after converting it practically completely into saturated esters by hydrogenation in presence of a nickel catalyst at 170–180°. In the direct fractionation of the "liquid" esters, the first fraction collected (which was usually about one-third of the material distilled) was not submitted to further refractionation, but was oxidised in acetone solution with potassium permanganate in order, as far as possible, to remove all unsaturated esters. Consequently, as in the case of a rat body fat [Banks *et al.*, 1933], the mean equivalent and unsaturation of the unsaturated esters could then be calculated, after the proportion and mean equivalent of the saturated esters (which remain unaltered during the oxidation) had been determined.

When the hydrogenated portion of the "liquid" esters was fractionally distilled, it was found in all cases to contain comparatively large proportions of methyl palmitate and, indeed, much larger amounts of this ester than were isolated by the oxidation procedure to which reference has just been made. This fact, in addition to giving an alternative quantitative estimate for palmitoleic acid present (see below), affords definite proof of the existence in the original "liquid" esters of an unsaturated derivative of the normal C_{16} series of acids.

The experimental data from which the final compositions of the mixed fatty acids were calculated, by means of the two latter alternatives (oxidation of the primary "liquid" ester fraction or fractionation of the hydrogenated "liquid" esters), are illustrated in Table II. This gives the results obtained with the abdominal and gizzard fats from the group of 24 hens which had been reared on a fish meal-free diet.

From the proportions and equivalents of the saturated esters obtained by permanganate-acetone oxidation of the fractions L 1, the weights of the various esters present therein were estimated to be as follows:

Estimated composition of methyl ester fractions L 1.

	Abdominal layer fat (g.)	Gizzard fat (g.)
Myristate	0·19	Trace
Palmitate	2·37	3·14
Palmitoleate	7·18	10·02
Oleate	11·58	11·57
Linoleate	8·21	9·19

Apart from this, the calculation of the components of the various ester fractions followed the customary procedure, and it may suffice to record here the percentage compositions found for the respective "solid," "liquid" and "hydrogenated liquid" groups of fatty acids (Table III).

Table IV gives the corresponding data for the total component acids of the two fats, derived from a combination of the values found for the "solid" acids and for the "liquid" acids by direct ester fractionation (and determination of saturated esters in the lowest-boiling "liquid" ester fraction).

Table III. *Estimated percentage compositions of the "solid," "liquid" and "hydrogenated liquid" acids.*

	Abdominal layer fat % (wt.)	Gizzard fat % (wt.)
"Solid" acids S:		
Myristic	0·3	0·2
Palmitic	73·7	73·1
Stearic	22·0	22·7
Oleic	4·0	4·0
Unsaponifiable	Trace	Trace
"Liquid" acids L:		
Myristic	0·2	Trace
Palmitic	3·2	3·6
Palmitoleic	7·7	11·0
Oleic	55·9	51·6
Linoleic	31·9	33·1
C_{20-22} unsaturated	0·8	0·4
Unsaponifiable	0·3	0·3
"Hydrogenated liquid" acids HL:		
C_{16}	13·4	14·6
C_{18}	85·6	84·2
C_{20-22}	0·9	0·8
Unsaponifiable	0·1	0·4

Table IV. *Summarised data for component fatty acids of original abdominal and gizzard fats.*

Acid	Solid acids S	Liquid acids L	Total	Fatty acids (excluding unsaponifiable matter) % (wt.)	% (mol.)
	Abdominal layer fat.				
	(31·8 %)	(68·2 %)			
Myristic	0·11	0·14	0·25	0·3	0·3
Palmitic	23·42	2·15	25·57	25·6	27·3
Stearic	7·00	—	7·00	7·0	6·7
Palmitoleic	—	5·27	5·27	5·3	5·7
Oleic	1·27	38·13	39·40	39·4	38·2
Linoleic	—	21·77	21·77	21·8	21·3
C_{20-22} unsaturated	—	0·57	0·57	0·6	0·5
Unsaponifiable	—	0·17	0·17	—	—
	Gizzard fat.				
	(31·1 %)	(68·9 %)			
Myristic	0·05	Trace	0·05	0·1	0·1
Palmitic	22·73	2·46	25·19	25·2	26·9
Stearic	7·06	—	7·06	7·1	6·8
Palmitoleic	—	7·58	7·58	7·6	8·1
Oleic	1·25	35·55	36·80	36·9	35·6
Linoleic	—	22·81	22·81	22·8	22·3
C_{20-22} unsaturated	—	0·28	0·28	0·3	0·2
Unsaponifiable	0·01	0·22	0·23	—	—

Table V gives the corresponding figures for the mixed fatty acids, when the increments of acids in the "solid" group are combined with those for the "hydrogenated liquid" acids. In this case allowance has been made for the slight alteration in the proportions of the two groups, due to the proportionate increase in weight of the "liquid" acids as a result of hydrogenation.

Since hydrogenation of an unsaturated acid gives rise to the same number of molecules of the corresponding saturated acid, and since nearly all the

Table V. *Summarised data for combination of "solid" acids with "hydrogenated liquid" acids of abdominal and gizzard fats.*

Acid	Solid acids S	Liquid acids L	Total	Fatty acids (excluding unsaponifiable matter) % (wt.)	% (mol.)
		Abdominal layer fat.			
	(31·7 %)	(68·3 %)			
Myristic	0·11	—	0·11	0·1	0·1
Palmitic	23·35	9·18	32·53	32·5	34·9
Stearic	6·98	58·45	65·43	65·5	63·3
Oleic	1·26	—	1·26	1·3	1·2
C_{20-22}	—	0·59	0·59	0·6	0·5
Unsaponifiable	—	0·08	0·08	—	—
		Gizzard fat.			
	(30·9 %)	(69·1 %)			
Myristic	0·05	—	0·05	0·1	0·1
Palmitic	22·59	10·06	32·65	32·7	35·1
Stearic	7·01	56·36	63·37	63·6	61·4
Oleic	1·24	1·86	3·10	3·1	3·0
C_{20-22}	—	0·54	0·54	0·5	0·4
Unsaponifiable	0·01	0·28	0·29	—	—

palmitic acid in the original fats appears in the "solid" group which is common to both series of analyses, a practically independent value for the palmitoleic acid present can be derived by combining the molar percentages of palmitic acid in Table V with those of Table IV. The stearic acid present in the original fat is also entirely segregated in the "solid" group and (with the minor proportions of myristic and C_{20-22} acids observed in the results given in Table V) the acids remaining over must originally have been C_{18} unsaturated acids. The molar percentage of these is therefore now determined by difference (the relative proportions of oleic and linoleic acids being calculated on the basis of those observed in the C_{18} unsaturated esters actually obtained in the course of the analysis by the direct fractionation of the "liquid" esters). The corresponding deduced molar (and thence weight) percentages of the acids present in the original fats, thus derived from the results of the analysis of the "hydrogenated liquid" esters, are given in Table VI, and it will be seen that the palmitoleic acid figures in this table and those in Table IV are in good agreement.

Table VI. *Alternative calculation of component acids from the palmitic acid figures before (Table IV) and after (Table V) hydrogenation.*

Acid	Abdominal layer fat % (mol.)	whence % (wt.)	Gizzard fat % (mol.)	whence % (wt.)
Myristic	0·1	0·1	0·1	0·1
Palmitic	27·3	25·6	26·9	25·3
Stearic	6·7	7·0	6·8	7·1
Palmitoleic	7·6	7·0	8·2	7·6
Oleic	37·1	38·4	35·4	36·6
Linoleic	20·7	21·3	22·2	22·8
C_{20-22} unsaturated	0·5	0·6	0·4	0·5

We have examined several of the individual ester fractions obtained in order to satisfy ourselves as to the identity of individual acids present.

(*a*) The fatty acids from the residual ester fractions L 7, after removal of unsaponifiable matter, were brominated in dry ethereal solution, when a very small amount of an insoluble bromo-addition product was precipitated, which was insoluble in boiling benzene and which decomposed without melting at about 220–230°. These properties are characteristic of the polybromo-addition products of highly unsaturated acids of the C_{20} or C_{22} series.

(*b*) Two fractions (L 4 and L 5) of "liquid" esters which appeared to consist wholly of C_{18} unsaturated esters were examined, after hydrolysis, with reference to the unsaturated acids present:

The acids (2·14 g.) from L 4 yielded no bromo-addition products insoluble in ether, but gave 0·42 g. of a substance, sparingly soluble in light petroleum, which, after crystallisation from the latter solvent, melted at 114° and showed no depression in melting-point when mixed with the tetrabromostearic acid isolated in the same manner from the mixed acids of cottonseed oil.

The potassium salts of the acids from L 5 were oxidised with dilute ice-cold alkaline permanganate according to Lapworth and Mottram [1925, 1]. From the products of oxidation there were isolated: (i) a tetrahydroxystearic acid (Found: C, 62·0; H, 10·0 %. $C_{18}H_{36}O_6$ requires: C, 62·1; H, 10·3 %) sparingly soluble in hot, and insoluble in cold, water, M.P. 168–171° (unchanged on admixture with the tetrahydroxystearic acid, M.P. 173°, obtained from the linoleic acid of cottonseed oil); and (ii) the dihydroxystearic acid, M.P. 130–131° (unchanged when mixed with an authentic specimen from ordinary oleic acid).

(*c*) As already stated, the saturated acids present in the lowest-boiling fractions resulting from redistillation of the fractions L 1 were exhaustively examined by fractional crystallisation in several cases. Several crops of crystals were obtained, the least soluble of which melted at 60·5–62·0° (unchanged when mixed with palmitic acid), whilst the most soluble acids melted at 46–47°; since this melting-point was raised to 52·5–55·5° by admixture with palmitic acid and to 47·5–50·0° by admixture with myristic acid, whilst addition of lauric acid depressed it to below 35°, it is clear that the only saturated components present were the two former acids.

(*d*) Identification of the saturated C_{16} acid produced during hydrogenation of the "liquid" esters was also attempted, but the lowest-boiling ester fractions (HL 1, equivalent 279–280) already contained much stearate, with the result that complete isolation of palmitic acid could not be effected. However, crystallisation from alcohol of the mixed acids (2·77 g.) from an ester fraction HL 1 gave a least soluble crop of crystals (1·55 g., M.P. 61–63°) and a second crop (0·73 g.) which melted sharply at 55°. The latter crop, the melting-point of which was raised on admixture with an equal weight of either palmitic or stearic acid, was evidently the characteristic palmitic-stearic acid "eutectic" mixture, whilst the first crop was similarly shown by mixed melting-point tests to be a mixture of palmitic with an excess of stearic acid. No indication was obtained of the presence of any acids other than palmitic and stearic.

(*e*) Finally, after the quantitative analyses had been completed, a mixture of the abdominal (500 g.) and gizzard (100 g.) fats from the hens fed on a fish meal-free diet was hydrolysed and submitted to the lead salt separation. The "liquid" acids were converted into methyl esters, and the lowest-boiling fractions were refractionated several times in order to obtain a mixture of esters as free as possible from C_{18} unsaturated components.

The specimen finally obtained (14·8 g., sap. equiv. 270·5, I.V. 80·3) was submitted to oxidation (and re-oxidation) in acetone solution with potassium permanganate. Unchanged saturated esters (4·02 g., sap. equiv. 264·2, I.V. 2·8)

were recovered, which indicated that the ester fraction oxidised was composed of saturated (4·0 g.), palmitoleic (8·7 g.) and C_{18} unsaturated (2·1 g.) esters.

The acidic products from the two oxidations were united, completely hydrolysed, and the mixture of mono- and di-basic acids (9·1 g.) was separated by cooling their light petroleum solution at 0°. The insoluble crystalline dibasic acids (6·2 g.) were crystallised from water and finally from ethyl acetate, when the product (3·1 g.) melted at 95–98°[1] (unchanged on admixture with azelaic acid) and had an equivalent of 93·2 (azelaic acid requires 94·0). The maximum possible yield of azelaic acid from the 2·1 g. of C_{18} unsaturated esters present in the substance oxidised is about 1·4 g., so that azelaic acid must have been produced in quantity from the palmitoleic ester. Moreover, the material (2·9 g.) which remained in solution in light petroleum was recovered and distilled at atmospheric pressure, when a distillate (1·4 g.), which came over at 220–225°, and possessed an equivalent of 133·0, was obtained (*n*-heptanoic acid, mol. wt. 130, boils at 223°). The residue from this distillation was solid at the ordinary temperature and was probably mainly azelaic acid, which is not completely insoluble in ice-cold light petroleum.

The palmitoleic acid of the hen fats is therefore the same as that which has been identified in various marine animal oils, and possesses the constitution $CH_3.[CH_2]_5.CH:CH.[CH_2]_7.COOH$.

It has been necessary to go into the foregoing details in consequence of the somewhat unusual character of the mixed fatty acids of the bird fats; but we may now proceed to tabulate the final results obtained for the abdominal, gizzard and neck fats of the two groups of birds. Table VII shows the data for

Table VII. *Component fatty acids of depot fats from group of 24 Light Sussex hens (age 7 months).*

Acid	Weight percentages			Molar percentages		
	Abdominal	Gizzard	Neck	Abdominal	Gizzard	Neck
(i) Calculated from the direct analysis.						
Myristic	0·3	0·1	0·3	0·3	0·1	0·3
Palmitic	25·6	25·2	26·7	27·3	26·9	28·4
Stearic	7·0	7·1	5·9	6·7	6·8	5·7
Palmitoleic	5·3	7·6	6·6	5·7	8·1	7·1
Oleic	39·4	36·9	39·0	38·2	35·6	37·6
Linoleic	21·8	22·8	21·2	21·3	22·3	20·6
C_{20-22} unsaturated	0·6	0·3	0·3	0·5	0·2	0·3
(ii) Calculated from the results of analysis of the "hydrogenated liquid" esters.						
Myristic	0·1	0·1	—	0·1	0·1	—
Palmitic	25·6	25·3	—	27·3	26·9	—
Stearic	7·0	7·1	—	6·7	6·8	—
Palmitoleic	7·0	7·6	—	7·6	8·2	—
Oleic	38·4	36·6	—	37·1	35·4	—
Linoleic	21·3	22·8	—	20·7	22·2	—
C_{20-22} unsaturated	0·6	0·5	—	0·5	0·4	—

the fats from the group of hens whose diet included no fish meal, whilst Table VIII shows the corresponding figures for the fats from the 12 hens which received 7 % of fish meal in their diet.

[1] A small amount of suberic acid (M.P. 132–136°, mixed M.P. 134–136°) was present with the azelaic acid; this was probably formed by further oxidation of a little dihydroxypalmitic acid produced during the initial oxidation [*cf.* Lapworth and Mottram, 1925, 2].

Table VIII. *Component fatty acids of depot fats from group of 12 Light Sussex hens (age 2 years).*

Acid	Weight percentages			Molar percentages		
	Abdominal	Gizzard	Neck	Abdominal	Gizzard	Neck
(i) Calculated from the direct analysis.						
Myristic	1·2	0·6	1·2	1·5	0·8	1·4
Palmitic	24·0	25·4	24·5	25·5	27·0	26·1
Stearic	4·1	4·2	4·2	3·9	4·0	4·0
Palmitoleic	6·7	7·1	6·9	7·2	7·7	7·4
Oleic	42·5	43·0	42·8	41·0	41·5	41·2
Linoleic	20·8	18·4	20·4	20·3	17·9	19·9
C_{20-22} unsaturated	0·7	1·3	Trace	0·6	1·1	Trace
(ii) Calculated from the results of analysis of the "hydrogenated liquid" esters.						
Myristic	0·9	0·3	—	1·1	0·4	—
Palmitic	24·0	25·4	—	25·5	27·0	—
Stearic	4·1	4·2	—	3·9	4·0	—
Palmitoleic	8·5	8·0	—	9·1	8·6	—
Oleic	41·3	43·2	—	39·9	41·7	—
Linoleic	20·3	18·5	—	19·7	18·0	—
C_{20-22} unsaturated	0·9	0·4	—	0·8	0·3	—

Summary and discussion.

The present work has shown that hen body fats possess specific characteristics which distinguish them from those of quadrupeds and also from those of fishes or marine mammals. They fall into one of the two broad groups of depot fats which are found in non-aquatic animals [*cf.* Banks *et al.*, 1933], namely, the class in which the component fatty acids contain only about 30–35 % of saturated acids. They are definitely not of the more saturated type found in the pig, ox, sheep, horse, *etc.*, in which the proportion of stearic acid is variable but usually considerable. In the hen depot fats, about 65 % of the component acids belong to the C_{18} series, but these are mainly oleic (35–38 %) and linoleic (20–22 %) acids; no evidence of acids more unsaturated than linoleic has been observed in the C_{18} group.

The presence of unsaturated C_{20-22} acids (although in very small amounts), but still more the occurrence of notable proportions (7–8 %) of palmitoleic acid, causes these bird depot fats to possess features which are somewhat reminiscent of those of marine animal fats. Indeed, the component acids of the hen fats may be regarded as intermediate in character between those of the fats of land and marine animals, but they are much closer to such fats as those of the rodents than to those of aquatic fauna.

It may be pointed out that palmitoleic acid (apart from its presence in the fats of aquatic animals) has only been reported up to the present in hen and rat body fats and in the fats of diphtheria bacilli [Chargaff, 1933] and of lycopodium spores [Riebsomer and Johnson, 1933].

The presence of 65–68 % of unsaturated acids in the component acids of the depot fats of an animal with so high a body temperature as the hen affords another interesting example of the fact that body temperature is by no means the sole determining factor of the relative saturation of depot fats. At the same time, the almost identical compositions of the fats from three different parts of the body of the birds, one of these being situated much farther from the outer skin than the others, may be contrasted with the progressive change in relative saturation on passing, for example, from the perinephric fat of the pig to the fat

lying nearest to the skin; and here it may well be that the more uniform body temperature of the feathered animal leads to the absence of any regional difference in depot fat composition. The influence of the body temperature is thus to be regarded as a secondary factor which may modify the composition of depot fat; but the specific type of the latter seems to depend primarily on the biological species.

Amongst the saturated acids of the hen fats, myristic acid is present in little more than traces, and stearic acid forms only 5–7 % of the total component acids. It is very interesting to observe however that, despite the specific features which seem to distinguish hen depot fats, their palmitic acid content is exactly of the same order (25–30 mol. %) as that found in practically all land animal fats, whether relatively saturated or unsaturated, studied up to the present. It is perhaps well to emphasise once more the regularity with which palmitic acid falls within these limits in the depot fats of land animals of widely varying types, and also to point out that a palmitic acid content of 25–30 % is practically never met with in the fats of marine animals and is only approached in very few instances in vegetable seed fats (in fruit coat fats the presence of even higher proportions of palmitic acid is of course less uncommon [Hilditch, 1933]).

Finally, we have shown that the occurrence of 7–8 % of palmitoleic acid in the hen fats is not dependent on the diet received by the birds. Within the limits of the experimental analytical error, all the six depot fats are closely similar. The only general differences between the two groups are that the proportions of stearic and linoleic acids are possibly slightly lower, and those of myristic, oleic and the C_{20-22} acids perhaps somewhat higher, on the whole, in the fats from the older birds (whose diet included fish meal) than in those of the others. This observation is indeed probably of greater interest in relation to the marked difference in age of the two groups of birds (7 months and 2 years respectively) than to any slight difference in their diets. The characters of the depot fats are the same both in young and in mature birds, fed on similar rations.

All the foregoing observations of course refer to birds which have received a diet of which fat formed only a minor proportion (4 % or less).

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REFERENCES.

Banks and Hilditch (1932). *Biochem. J.* **26**, 298.
—— —— and Jones (1933). *Biochem. J.* **27**, 1375.
Bömer and Merten (1922). *Z. Unters. Nahr. Genussm.* **43**, 101.
Chargaff (1933). *Z. physiol. Chem.* **218**, 223.
Grossfeld (1930). *Z. Unters. Lebensm.* **60**, 64.
—— (1931). *Z. Unters. Lebensm.* **62**, 553.
Hilditch (1933). *J. Soc. Chem. Ind.* **52**, 169T.
Lapworth and Mottram (1925, 1). *J. Chem. Soc.* **127**, 1628.
—— —— (1925, 2). *J. Chem. Soc.* **127**, 1987.
Riebsomer and Johnson (1933). *J. Amer. Chem. Soc.* **55**, 3352.

CXI. FERMENTATION OF SUGAR BY THE ROOT NODULE BACTERIA[1].

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The fermentation of carbohydrates by the root nodule bacteria is a question of great interest, considering that the ability to fix atmospheric nitrogen must necessarily be dependent on the ability to ferment and utilise suitable carbon compounds. The solution of this question might possibly throw some light on the complicated problem of the mechanism of nitrogen fixation.

Although a vigorous fixation of nitrogen occurs when the nodule bacteria are grown in symbiosis with the appropriate host plant, recent investigations have lent additional support to the view that these bacteria are not capable of fixing nitrogen when grown apart from the host. Nor does any fixation occur when the nodules as such are separated from the host plant and cultivated on a synthetic medium, so that the power to fix atmospheric nitrogen cannot be attributable to any special difference in the structure of endonodular bacteria. In our opinion, all this leads to the assumption that the nodule bacteria receive from their host plant some particularly suitable form of carbon nutrition—probably labile carbohydrates, trioses or hexoses—which enables them to carry out the fixation process.

The utilisation of various carbon compounds by the nodule bacteria has been the subject of numerous and extensive investigations. In this respect, our knowledge has been particularly widened by the work of Baldwin and Fred [1927]. These investigations show that nodule bacteria actually utilise a great variety of carbohydrates and related compounds, although different strains differ considerably in this respect.

The decomposition of sugars by the nodule bacteria generally takes place very slowly. Aldohexoses seem to be more readily fermentable than fructose. The characteristics of the fermentation differ considerably for different strains, some strains causing the reaction of the medium to become acid, others alkaline. Very little is known, however, of the fermentation products formed, with the exception of the gum or slime which is obviously produced from carbohydrates.

As far as we know, the actual process of the fermentation of sugars by the nodule bacteria is totally unexplored. This is largely due to the fact that by the old technique hitherto employed—inoculation of the substrate solution with small amounts of the organism in question—the isolation and accurate determination of different fermentation products is scarcely possible owing to the very slow rate of the decomposition. We have therefore used in the present work the technique employed in this laboratory for several years, *i.e.* we have cultivated the organism separately on suitable media and used heavy suspensions

[1] A preliminary note on the subject appeared in the *Acta Chemica Fennica*, B 4, 62 (1932).

of the cell-mass thus obtained to act upon the substrate. The advantages of this method are obvious. The increased rate of sugar breakdown renders possible the identification and determination of the end-products, whilst the mechanism of the fermentation can be substantially elucidated by interrupting the process at different stages and determining the intermediate products present.

Experimental.

The bacterial species used was a laboratory strain of *Rhizobium trifolii* isolated from red clover and carried in stock for several years.

For the production of bacterial mass, 300–500 slant gelatin test-tubes were used at a time. The nutrient medium was prepared as follows:

Glucose	5·0 g.
Asparagine	2·5 g.
Gelatin	130 g.
Pea-extract	200 ml.
Tap-water	to 1000 ml.

The inoculated cultures were maintained at room temperature for 4 days, after which the bacterial mass was washed off with sterile water. This was done in an inoculation room where all necessary apparatus had been previously sterilised with a Hg-lamp. The suspension was centrifuged clear, and the bacterial mass was washed with warm sterile water. 100 test-tubes produced approximately 1·7 g. of bacterial mass which was practically free from gum.

A total of 20 fermentation experiments was carried out. Calcium lactate was used as substrate in two experiments and calcium butyrate in one, whilst glucose (1–6 g.) was used in all other experiments. In most cases, calcium carbonate was added to neutralise the acids formed during the fermentation. The fermentations were carried out under anaerobic conditions[1] attained by filling the fermentation flasks either with carbon dioxide or nitrogen. All fermentations were carried out at a temperature of 37°. The gases produced were collected and measured over mercury.

The evolution of gas set in, as a rule, about 30 hours after the addition of the bacterial suspension. A considerable portion of the gas was hydrogen.

The destruction of sugar was not complete. In several experiments only about one-half of the glucose originally present was fermented. In very few cases the destruction of sugar amounted to 75 % or more of the original. Sugar determinations were made by Bertrand's method, and the reducing sugar was identified as unfermented glucose by means of its phenylosazone.

After the fermentation had come to an end, the fermented liquid was tested for bacterial purity both microscopically and using gelatin plates. Analyses of the fermentation products were carried out in the manner generally employed in this laboratory.

In one experiment, sterile nodules were used instead of bacterial suspension as the fermenting agent. The nature of the fermentation thereby caused was similar to that caused by the bacterial suspensions.

Results.

It appeared that the relative proportions of fermentation products were closely dependent on the duration of the experiment. During the first stages of the process, considerable amounts of inactive lactic acid were formed from

[1] A preliminary experiment was also made aerobically. The presence of butyric acid in the fermented liquid was noted, but no accurate analysis of the end-products was made.

glucose. Lactic acid is, however, slowly fermented further with the production of butyric acid which is one of the end-products of the fermentation. In addition to lactic and butyric acids, small amounts of acetic acid, ethyl alcohol, carbon dioxide and hydrogen are also formed. The molecular ratio of butyric acid to carbon dioxide and hydrogen is: $C_4H_8O_2:CO_2:H_2=1:2:2$. The fermentation of glucose by the root nodule bacteria is thus a butyric acid fermentation.

Table I shows the results of a series of experiments. In each experiment equal amounts of glucose and $CaCO_3$ and equal amounts of the same bacterial mass were used, the duration of the experiments varying from 90 to 306 hours.

Table I.

Each flask contained 4 g. glucose, 5 g. $CaCO_3$, 2·2 g. moist bacterial mass, 250 ml. tap-water and was filled with nitrogen gas.

Exp. No.	10	11	12
Duration of experiment, hours	90	142	306
Glucose fermented, %	48·2	50·6	84·35
Alcohol, % of fermented glucose	5·25	2·4	6·55
Lactic acid, % of fermented glucose	22·6	18·2	3·0
Butyric acid, % of fermented glucose	24·2	27·3	35·45
Acetic acid, % of fermented glucose	2·9	4·05	Traces

For comparison, results from some other experiments are given in Table II.

Table II.

Exp. No.	7	9	14*
Duration of experiment, hours	96	90	90
Glucose fermented, %	72·2	49·2	29·0
Alcohol, % of fermented glucose	Traces	3·8	Not determined
Lactic acid, % of fermented glucose	40·2	21·4	26·8
Butyric acid, % of fermented glucose	13·0	31·8	30·5
Acetic acid, % of fermented glucose	4·2	Traces	5·1
CO_2, % of fermented glucose	16·0	Not determined	Not determined
H_2, % of fermented glucose	Not determined	Not determined	1·5

* The bacterial mass used in this experiment was somewhat too old and had lost a great part of its activity.

Identification and determination of different fermentation products.

Lactic acid. Quantitative determination of lactic acid was made using the Hirsch-Kauffman [1924] modification of the Fürth-Charnass method.

For identification, lactic acid was isolated from the fermentation liquid as zinc salt after the volatile acids had been distilled off with steam. The product was twice recrystallised from hot water and gave on analysis values consistent with the formula $(C_3H_5O_3)_2Zn, 3H_2O$ for the zinc salt of *dl*-lactic acid. (Found: H_2O, 18·19; ZnO, 26·87 %. $(C_3H_5O_3)_2Zn, 3H_2O$ requires H_2O, 18·18; ZnO, 27·37 %.)

Alcohol was determined according to Kuriloff [1897].

In order to identify the volatile neutral substance produced in the fermentation, the fermented liquid was subjected to steam-distillation at about p_H 8. The distillate was then acidified with sulphuric acid to p_H 2. The volatile substance was again distilled and oxidised with potassium dichromate and sulphuric acid in an autoclave at 110°. The acids thereby formed were determined according to Virtanen [1923] and Virtanen and Pulkki [1928]. It appeared that the oxidation yielded only acetic acid and that, consequently, the volatile fermentation product was ethyl alcohol.

Example. Determination of the original alcohol content according to Kuriloff gave 0·222 g. alcohol in the distillate. From this amount, 46·4 ml. of 0·1 *N* acetic acid were formed on oxidation, corresponding to 0·214 g. ethyl alcohol. The value for the semi-distillation was 36·3 %.

Carbon dioxide. In Exp. 7 the fermentation flask contained:

Glucose	3 g.
$CaCO_3$ (pure)	3·5 g.
Bacterial mass	3·0 g.
CO_2-free tap water	300 ml.

All air in the flask was replaced by CO_2-free nitrogen. The gases evolved were passed into KOH. After completed fermentation the flask was kept in a boiling NaCl solution for 1½ hours, a slow stream of nitrogen being passed through the apparatus. The weight of CO_2 obtained was 0·665 g.

On the basis of the figures presented in Table II the following calculation can be made:

0·091 g. acetic acid	liberates	0·0333 g.	CO_2 from $CaCO_3$
0·871 g. lactic acid	,,	0·213	,,
0·281 g. butyric acid	,,	0·070	,,

Total amount of CO_2 liberated by acids = 0·3163 g.

Assuming that in the production of each molecule of acetic acid one molecule of CO_2 is split off—for 0·091 g. acetic acid 0·0667 g. CO_2—the amount of CO_2 formed in the butyric fermentation will be:

$$0{\cdot}665 - (0{\cdot}3163 + 0{\cdot}0667) = 0{\cdot}282 \text{ g.}$$

In the above experiment 0·281 g. butyric acid was formed, the ratio butyric acid : carbon dioxide being thus 1 : 2.

Attempts to measure the CO_2 production directly, using strong buffer solutions, failed.

Hydrogen. In Exp. 14, all air was carefully removed from the fermentation apparatus by means of carbon dioxide. The gases produced by fermentation were first led into a mercury burette and thereafter into a burette filled with 40 % KOH. The amount of hydrogen was 162 ml., *i.e.* 13·3 mg. of hydrogen at N.T.P., against 12·0 mg., calculated from the amount of butyric acid formed (0·265 g.).

Succinic and formic acids and glycerol. Succinic acid was determined only in Exp. 1, its amount being found to be 1·2 % of the glucose fermented. This small amount might possibly have been formed from the bacterial mass.

Glycerol is not produced by the nodule bacteria. The same is true of formic acid. Determinations according to Eds [1924–25] gave negative results.

Fermentation of lactic acid by the nodule bacteria.

This was studied in Exps. 6 and 15, using calcium lactate as substrate. It appeared that lactic acid itself is fermented very slowly. Thus, in Exp. 15 only 5 % of the lactic acid was decomposed in 4 days. In Exp. 6, 1·583 g. of pure lactic acid (neutralised with $CaCO_3$) were used together with 1·5 g. of moist bacterial mass. From this amount 1·077 g. were recovered after 11 days. The extent of destruction was thus only 28 %. Amongst the fermentation products were found 0·175 g. of acetic acid and 0·071 g. of butyric acid.

Recovery and identification of unfermented glucose in the fermentation liquid.

Exp. 18. The fermentation flask contained:

Glucose	3 g.
$CaCO_3$	3 g.
Bacterial mass	3·5 g.
Tap-water	250 ml.

The fermentation was carried out in an atmosphere of carbon dioxide. The evolution of gas ceased after 10 days. The volume of the gas was not measured.

The amount of glucose fermented was 2·765 g. or 92·2 %.

After completed fermentation the liquid was centrifuged, acidified with acetic acid and concentrated under reduced pressure to a volume of 95 ml. 30 ml. of the concentrate, containing 68 mg. sugar (as glucose), were heated on the water-bath with some $CaCO_3$ for half an hour. After cooling, 0·2 g. of fresh baker's yeast was added and the flask connected with a 10·0 ml. gas-burette. Temperature 37°. 17·5 ml. of gas were obtained in 23 hours and the fermented liquid did not reduce Fehling's solution. The 68 mg. glucose originally present in the concentrate would have given rise to about 33 mg. or 16·5 ml. of CO_2.

From the remaining 65 ml. of the concentrate the protein substances were removed by $HCl + HgCl_2$, the mercury was precipitated with H_2S and the hydrogen sulphide removed by a stream of air. The solution was then brought with NaOH to about p_H 5 and concentrated *in vacuo* to a volume of 30 ml. and treated with phenylhydrazine hydrochloride and sodium acetate on the water-bath. The phenylosazone formed was washed with hot water and further purified by recrystallisation from 50 % alcohol. It consisted of yellow needles which melted sharply at 205°.

The reducing substance in the fermentation liquid was thus unfermented glucose.

Experiment in which the bacterial mass was grown on liquid medium.

The growth of the nodule bacteria on liquid media is generally very slow, and it is therefore difficult to obtain considerable quantities of the bacteria from liquid cultures. An addition of mould-extract, however, promotes the growth of the nodule bacteria to a surprisingly great extent. The mould-extract was prepared by growing *Aspergillus niger* in suitable media and extracting the mycelium obtained with hot water at p_H 7.

The following experiment was, however, made with bacterial mass grown in an ordinary culture medium of the following composition:

KNO_3	0·5 g.	$FeCl_3$	Traces
K_2HPO_4	0·5 g.	$MnSO_4$	Traces
$MgSO_4, 7H_2O$ ...	0·2 g.	Yeast-extract ...	200·0 ml.
NaCl	0·2 g.	Tap-water ...	to 1000·0 ml.

After incubation for 7 days, the bacterial mass was centrifuged off, washed and suspended in a fermentation flask containing:

Glucose	3 g.
$CaCO_3$	3 g.
Tap-water	250 ml.

The weight of the bacterial mass obtained was 2 g.

The experiment was carried out in an atmosphere of CO_2 for 7 days. The amount of gas produced was 425 ml. The results were:

Glucose fermented ...	1·07 g. = 35·7 %	
Alcohol	0·039 g. = 3·6 %	of ferm. glucose
Butyric acid	0·443 g. = 41·5 %	,,
Lactic acid	0·058 g. = 5·5 %	,,

Other products were not determined.

Experiments with sterile nodules.

Twenty-four pea-plants, inoculated with *Rh. leguminosarum*, were grown in six Woulff-flasks in quartz sand under aseptic conditions[1]. After 2 months, the nodules were aseptically cut off from the roots, crushed in a sterile mortar, centrifuged off, washed with sterile water and suspended in a solution containing

Glucose	3 g.
$CaCO_3$	3 g.
Tap-water	250 ml.

The weight of the moist nodule-mass was 5 g. Development of gas ceased after 67 hours. The volume of gas was not measured. Plate culture on gelatin remained clear for over 3 days. Results:

Glucose fermented ...	0·945 g. = 31·5 %	
Alcohol	0·079 g. = 8·4 %	of ferm. glucose
Lactic acid	0·160 g. = 17·0 %	,,
Acetic acid	0·115 g. = 12·2 %	,,
Butyric acid	0·185 g. = 19·6 %	,,

A comparison of the above results with those given in Tables I and II shows that the fermentation caused by separated nodules is, on the whole, very similar to that caused by bacterial suspensions, though in the former case slightly more acetic acid and alcohol were formed.

Catalase content of Rh. trifolii.

Determination of the catalase content of *Rh. trifolii* gave the following results:

$$k = 0{\cdot}00337, \text{ kat. v.} = \frac{0{\cdot}00337}{1350 \times 10^6} = 0{\cdot}0025 \times 10^{-9}.$$

The catalase content of the nodule bacteria is thus comparatively low. For comparison it may be mentioned that Virtanen and Karström [1925] and Virtanen and Winter [1928] found the following values for the catalase content of certain common micro-organisms:

B. fluorescens liquefaciens	$0{\cdot}08 \times 10^{-9}$
B. pyocyaneum	$0{\cdot}41 \times 10^{-9}$
Acetobacter suboxydans	$0{\cdot}01 \times 10^{-9}$
B. coli (10 strains)	$0{\cdot}004$–$0{\cdot}02 \times 10^{-9}$
B. acidi propionici	$2{\cdot}2 \times 10^{-9}$

[1] The method thereby employed has been described by Virtanen *et al.* [1933].

SUMMARY.

Fermentation of glucose by the root nodule bacteria was studied using heavy suspensions of *Rh. trifolii*, grown separately on a pea-extract gelatin medium.

The fermentation thereby caused is of the butyric type. During the early stages of the process considerable amounts of *dl*-lactic acid are formed from glucose. Lactic acid is, however, slowly fermented further with the production of butyric acid which is one of the end-products. In addition to lactic and butyric acids, carbon dioxide, hydrogen and small amounts of acetic acid and ethyl alcohol are also formed. The molecular ratio of butyric acid to carbon dioxide and hydrogen is $C_4H_8O_2 : CO_2 : H_2 = 1 : 2 : 2$.

Similar fermentation of glucose is also caused by crushed nodules from plants grown in sterile quartz sand cultures.

An aqueous extract of mould mycelium was found to stimulate markedly the growth of *Rh. trifolii*.

REFERENCES.

Baldwin and Fred (1927). *Soil Sci.* **24**, 217.
Eds (1924–25). *J. Lab. Clin. Med.* **10**, 1, 59.
Hirsch-Kauffman (1924). *Z. physiol. Chem.* **140**, 25.
Kuriloff (1897). *Ber. deutsch. chem. Ges.* **30**, 741.
Virtanen (1923). *Soc. Scient. Fennica, Comm. Physico-Mathemat.* **1**, 36.
—— v. Hansen and Karström (1933). *Biochem. Z.* **258**, 106.
—— and Karström (1925). *Biochem. Z.* **161**, 9.
—— and Pulkki (1928). *J. Amer. Chem. Soc.* **50**, 3138.
—— and Winter (1928). *Biochem. Z.* **197**, 210.

CXII. THE METABOLISM OF NAPHTHALENE IN RABBITS.

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Interest in the metabolism of aromatic hydrocarbons has been stimulated by the recent work on the carcinogenic properties of certain members of this group of substances. But the problem of the metabolism of naphthalene is of especial interest because of the property which this substance possesses of being able to produce, when administered to rabbits in repeated doses, an opacity of the crystalline lens of the eye which, histologically, closely resembles human senile cataract [Bouchard and Charrin, 1886; Adams, 1930].

The work reported in the present paper forms part of a series of investigations directed towards the discovery of the mechanism by which naphthalene acts upon the organism so as to induce degenerative changes in the lens-proteins. At present nothing is known of this mechanism; it is still uncertain whether naphthalene or some derivative of it formed in the body acts directly on the lens, or whether the cataract is a secondary phenomenon resulting from some metabolic disturbance or toxic action upon a tissue remote from the eye. An essential step in the solution of this problem appeared to be to obtain some knowledge of the fate of ingested naphthalene. Therefore an attempt to identify the excretory products of naphthalene in the urine of rabbits has been made.

It is the purpose of the present paper to describe the isolation and identification of one metabolic product of naphthalene, *viz.* *l*-α-naphthylmercapturic acid; and to present evidence for the existence of a second metabolite, which has not been isolated, but which gives rise to naphthalene on acidification.

Experimental.

Buck rabbits weighing between 2 and 3 kg. and feeding on oats and cabbage were used exclusively. Naphthalene was dissolved in warm liquid paraffin and administered by means of a stomach tube. Doses of 1 or 2 g. were given to each rabbit and repeated at intervals of 2 or 3 days. The rabbits were kept in individual metabolism cages of suitable size, and the urine was collected separate from the faeces.

Colour tests for naphthols and glycuronic acid.

Certain authors [Igersheimer and Ruben, 1910; Edelfsen, 1905], on the basis of colour reactions applied to the urine excreted by dogs after naphthalene feeding, concluded that naphthalene was oxidised to naphthol in the body and was excreted in the urine as α- or β-naphthylglycuronate. Lesnick [1888] isolated a small amount of chemically pure α-naphthylglycuronate from dog's urine after the feeding of 12 g. of naphthalene. Penzoldt [1886] was unable to

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obtain positive tests for naphthols, but presented evidence for the presence of naphthoquinone. Baumann and Herter [1877] obtained no naphthol on distillation of acidified naphthalene urine and reported that with steam-distillation only unchanged naphthalene came over.

We have not succeeded in isolating either naphthols or naphthylglycuronate from rabbit urine after dosing with naphthalene, but we have obtained positive diazo and ferric chloride reactions when these tests were applied to the residue from an ether extract of acidified naphthalene urine after hydrolysis. We have also obtained positive naphthoresorcinol tests for glycuronic acid. However, when these tests are applied to so complex a mixture as urine, the results cannot be interpreted with certainty, and we are led to conclude that, although naphthols may be present as ethereal sulphates or glycuronates, the amount is small and they do not appear to be the chief metabolic product of naphthalene in the case of the rabbit.

A compound giving rise to naphthalene on acidification.

The urine of rabbits after naphthalene feeding contains a soluble compound which yields naphthalene on acidification. This was demonstrated in the following manner. The alkaline urine excreted by a rabbit during the 24 hours following a 2 g. dose of naphthalene was collected. It was divided into two equal portions, each portion being placed in a conical flask. One portion was made acid with HCl, the other was kept as control and both were heated on a boiling water-bath. Within a short time colourless crystals began to collect on the cool parts of the flask containing the acidified urine; in the control flask no crystals appeared. The crystals were collected, washed with dilute acid, dilute NaOH and water; they were dried and resublimed. Their melting-point was found to be 79·5°; a mixture of these crystals and naphthalene also melted at 79·5°. A picrate melting at 149·5° was prepared from them. Their identity as naphthalene was thus established.

Naphthalene was not present in the alkaline urine, since none was sublimed out on heating the control flask, but was rapidly formed on acidification from some soluble precursor present in the urine. Efforts to isolate such a substance have so far been unsuccessful. The observation described might be accounted for by the presence in the urine of some compound such as dihydro-β-naphthol, a soluble compound which is rapidly converted to naphthalene by mineral acids [Bamberger and Lodter, 1895], and which might conceivably be formed in the body as a product of naphthalene metabolism.

1-α-*Naphthylmercapturic acid.*

This compound is not present in the urine of normal rabbits, nor does it occur after the feeding of liquid paraffin alone. It was found in the urine of rabbits after naphthalene feeding and was isolated in the following manner. The urine excreted by three rabbits during the 48 hours following the administration of 2 g. naphthalene to each rabbit was collected and mixed. It was made just neutral to litmus with HCl and then conc. HCl was added in the proportion of 10 ml. of acid for 100 ml. urine. The acidified urine was allowed to stand for about $\frac{1}{2}$ hour after which it was twice extracted with an equal volume of chloroform. The chloroform layer was collected and transferred to a distillation flask. Most of the chloroform was distilled off on a water-bath, and when the extract had been reduced to a small volume, it was transferred to an evaporating dish and the remainder of the chloroform carefully evaporated off with gentle heating on the water-bath.

The dark-brown, gummy residue so obtained was dissolved in a small volume of absolute ethyl alcohol. The alcohol solution was filtered if necessary and then diluted with 0·5 *N* HCl until opalescent. After standing in the ice-chest overnight a considerable bulk of precipitate consisting of colourless crystals mixed with amorphous brown material settled out. After this had been filtered off a second crop of crystals was sometimes obtained by further dilution of the filtrate.

The precipitate, after washing with water, was extracted with saturated barium hydroxide solution. The filtered baryta extract was acidified with HCl, whereupon an impure crystalline product separated out. This material was filtered off, washed with water and dried on a porous plate. The impurities present at this stage, which imparted a pinkish-brown colour to the material, possessed solubilities similar to those of the desired substance; further purification was therefore a matter of some difficulty. For this purpose the dried material was treated with a few ml. of cold 2 *N* NaOH; at first all the material went into solution, but upon brisk stirring the sodium salt of the compound separated out. This precipitate was filtered off immediately on a sintered glass filter and washed with a few ml. 2 *N* NaOH. The filtrate, which was coloured dark-brown, contained most of the contaminating substances. The sodium salt was dissolved in water; upon acidification of the solution the acid was precipitated as colourless crystals. Treatment with 2 *N* NaOH was repeated if the product obtained was more than faintly coloured.

The final stages of the purification consisted in repeated recrystallisations from aqueous alcohol until a colourless product of constant melting-point was obtained.

Amounts of l-α-*naphthylmercapturic acid present in urine.* From the 24 hours' urine of a single rabbit after 1 g. dose of naphthalene 52 mg. of the pure compound were obtained; 260 mg. of the pure compound were isolated from the mixed 24 hours' urine of three rabbits after the administration of 1 g. of naphthalene to each rabbit. Yields of between 300 and 400 mg. of the pure compound were obtained from the mixed 48 hours' urine of three rabbits after the administration of 2 g. of naphthalene to each rabbit.

Properties of l-α-*naphthylmercapturic acid.* It is an easily crystallisable, colourless, odourless solid; needles are obtained from aqueous alcohol; M.P. 170·5–171° (uncorr.). It is insoluble in water and in dilute mineral acids; soluble in alcohol, chloroform and pyridine; less soluble in ether, and only slightly soluble in hot benzene and toluene; soluble in dilute NaOH, barium hydroxide, sodium bicarbonate and dilute ammonia; completely soluble in conc. H_2SO_4 and when the solution is warmed a clear green colour develops. The compound is an acid and leaves no residue on ashing. When heated with soda-lime ammonia is easily split off. Carbon, nitrogen and sulphur were shown to be present; the test for ethereal sulphates was negative.

Analysis. After drying *in vacuo* at 80° over P_2O_5 the following analytical values were obtained (Schoeller). (Found: C, 62·14, 62·22; H, 5·23, 5·38; S, 11·24, 11·16; N, 4·75, 4·64 %. $C_{15}H_{15}O_3NS$ requires C, 62·28; H, 5·23; S, 11·09; N, 4·84 %.)

Mol. wt. By the camphor method, 311 (Schoeller); theory requires 289.

Neutralisation equivalent. The substance was dissolved in neutral alcohol and titrated with *N*/50 NaOH using phenolphthalein as an indicator. Found: 292; $C_{15}H_{15}O_3NS$ as a monobasic acid requires 289. The compound is therefore a monobasic acid.

Optical activity. The compound is laevorotatory; a 1 % solution in absolute ethyl alcohol has $[\alpha]_D^{23°} -25°$.

Constitution of l-α-*naphthylmercapturic acid.*

Decomposition by acid. 0·669 g. of the substance was boiled for ¾ hour under reflux with 50 ml. dilute H_2SO_4 (12·5 % by volume). About half of the material remained undissolved and was removed by filtration of the hot solution. As the filtrate cooled slightly a small amount of crystalline material precipitated; this was also removed by filtration. The filtrate, while still warm, was made slightly alkaline to litmus by the cautious addition of concentrated ammonia. Upon cooling a precipitate appeared which separated out in crystalline form when the solution was allowed to stand in the ice-chest overnight. This precipitate was filtered off on a sintered glass filter; washed with water and dried at room temperature in the air; yield 0·183 g. This was recrystallised from hot water and a pure product in poor yield was obtained.

The recrystallised product was dried in air at 37° and had the following properties: insoluble in cold water, cold alcohol, ether, chloroform, and sodium bicarbonate solution; soluble in hot water, hot alcohol, dilute NaOH and dilute H_2SO_4; completely soluble in conc. H_2SO_4 and on warming the same green colour as is obtained with the parent substance developed. It had no definite melting-point but decomposed between 160 and 165°.

Analysis (Schoeller). (Found: C, 63·23, 63·08; H, 5·36, 5·36; S, 13·10; N, 5·44 %. $C_{13}H_{13}O_2NS$ requires C, 63·12; H, 5·30; S, 12·95; N, 5·66 %.)

A comparison of the empirical formula of this derivative with that of the parent substance showed that the two differed by C_2H_2O. It was therefore concluded that the effect of acid hydrolysis had been to split off one acetyl group from the molecule according to the equation:

$$C_{15}H_{15}O_3NS + H_2O \rightarrow C_{13}H_{13}O_2NS + CH_3COOH.$$

Acetyl estimation. The presence of one acetyl group in the molecule of the parent substance was proved by micro-acetyl estimation. (Found: 14·27, 14·21 % acetyl (Roth); theory requires 14·89 %.)

Decomposition by alkali. When boiled with dilute sodium hydroxide the compound was decomposed with the evolution of ammonia. Upon subsequent acidification of the solution a cloudiness appeared and after standing in the ice-chest, an oil separated out, which was soluble in alcohol and ether, gave a positive test for sulphur and had a characteristic unpleasant odour which suggested naphthylmercaptan. Since its low melting-point made it unsuitable for analysis, it was decided to oxidise it in the hope of obtaining a dinaphthyldisulphide. Naphthylmercaptans are easily oxidised by the oxygen of the air in the presence of dilute sodium hydroxide.

0·498 g. of the parent substance was suspended in 50 ml. of 6 % NaOH and boiled for ¾ hour under reflux. The solution was then cooled, mixed with 50 ml. water and aerated for 48 hours. The solution soon became turbid, and a colourless solid separated out during the aeration period. After aeration was complete the mixture was allowed to stand in the ice-chest overnight to insure complete precipitation; the precipitate was filtered off, washed with water, and recrystallised from aqueous ethyl alcohol. In this way a product of definite melting-point, suitable for purification and analysis, was obtained.

It was a crystalline substance melting at 89°; soluble in ether and hot alcohol. Analysis (Schoeller): (Found: C, 75·60; H, 4·26; S, 20·17 %. $C_{10}H_7.S.S.C_{10}H_7$ requires C, 75·44; H, 4·43; S, 20·15 %). This substance is therefore identified as αα-dinaphthyldisulphide (M.P. 91°), the melting-point of the ββ-form being 139°.

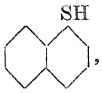
The fact that αα-dinaphthyldisulphide could be isolated when alkaline decomposition of the compound was followed by aeration proves that α-naphthylmercaptan was one of the products of alkaline hydrolysis of the compound $C_{15}H_{15}O_3NS$.

Formula. On the basis of the information obtained by the acid and alkaline hydrolyses of the compound a probable constitutional formula can be deduced.

One acetyl group is present in the molecule, and the amphoteric character of the deacetylated compound suggests that the acetyl group is combined with nitrogen. Since one of the oxygen atoms is known to belong to an acetyl group, the possibility that the compound is a sulphonic acid is ruled out, and the acidic properties of the substance must be attributed to the presence of a carboxyl group.

That the compound is a naphthalene derivative is proved by the identification of α-naphthylmercaptan as one of the products of alkaline decomposition. The facts that the naphthalene nucleus is linked to the rest of the molecule through sulphur and that the linkage is in the α-position are also established by this means.

The following fragments have therefore been identified:

COOH, $NH.CO.CH_3$, 

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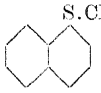
and of the whole molecule only C_2H_3 is left unaccounted for. On the basis of these facts the following constitutional formula is proposed:



This compound is an acetylated cysteine derivative, analogous to *p*-bromophenylmercapturic acid, which is excreted after bromobenzene feeding. So far as we are aware, it has not been previously described.

Discussion.

It has been shown that naphthalene is detoxicated by the rabbit partly by conjugation with cysteine and excretion as the mercapturic acid and partly by conversion into a soluble compound, not yet identified, which yields naphthalene on acidification. It is possible that other naphthalene derivatives, such as ethereal sulphates or phenolic substances are also excreted.

The evidence presented for the suggested constitutional formula of *l*-α-naphthylmercapturic acid has been confirmed by the synthesis of this compound, which is described in the following paper [Ing *et al.*, 1934].

Two interesting suggestions arise out of the observations here reported. In the first place conversion into the mercapturic acid may be a general method of detoxication of aromatic hydrocarbons in the animal body. Callow and Hele [1926] presented evidence that benzene as well as bromobenzene is excreted as the mercapturic acid, although the phenylmercapturic acid was not isolated. Secondly the fact that naphthalene requires cysteine for its detoxication suggests the possibility that the cataract which naphthalene produces may result from depriving the lens of cysteine. The β-krystallin of the lens is a cysteine-rich protein; glutathione and possibly free cysteine are present in the lens in relatively large amounts; furthermore the lens, which is an avascular tissue and

dependent on the aqueous humour for its supply of oxygen, is thought to have a specialised oxidation mechanism in which the cysteine-containing substances play an important rôle. It therefore seems likely that repeated doses of naphthalene, constituting a drain on the cysteine supplies in the body, might interfere with the metabolism of the lens by depriving it of cysteine; and thus the degenerative changes resulting in cataract might be induced.

SUMMARY.

An organic acid, $C_{15}H_{15}O_3NS$, identified as *l*-α-naphthylmercapturic acid, has been isolated from the urine of rabbits after naphthalene dosing; the isolation and identification are described.

A second compound, a soluble substance yielding naphthalene on acidification, was also found to be present; this has not been isolated.

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REFERENCES.

Adams (1930). *Brit. J. Ophthal.* **14**, 49.
Bamberger and Lodter (1895). *Liebig's Ann.* **288**, 74.
Baumann and Herter (1877). *Z. physiol. Chem.* **1**, 244.
Bouchard and Charrin (1886). *Compt. Rend. Soc. Biol.* **8**, 614.
Callow and Hele (1926). *Biochem. J.* **20**, 598.
Edelfsen (1905). *Arch. exp. path. Pharm.* **52**, 429.
Igersheimer and Ruben (1910). *Arch. Ophthal.* **74**, 467.
Ing, Bourne, and Young (1934). *Biochem. J.* **28**, 809.
Lesnick (1888). *Arch. exp. Path. Pharm.* **24**, 167.
Penzoldt (1886). *Arch. exp. Path. Pharm.* **21**, 34.

CXIII. SYNTHESIS OF *l*-α-NAPHTHYL-MERCAPTURIC ACID.

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In the preceding paper the isolation of an acid $C_{15}H_{15}O_3NS$ from the urine of rabbits fed with naphthalene was described and evidence presented which identified it with reasonable certainty as *l*-α-naphthylmercapturic acid, $C_{10}H_7S.CH_2.CH(NHAc).CO_2H$. In order to put this identification beyond doubt, the synthesis of this acid was attempted and has now been achieved.

The best method of synthesising mercapturic acids appeared to be the condensation of the appropriate aryldiazonium salt with acetylcysteine. Diazonium salts, ArN_2X, condense with thiol compounds to form $ArN_2S.R$, which decompose when heated in a suitable solvent, evolving nitrogen and forming thio-ethers, Ar.S.R [Kalle and Co., 1907]. Friedmann [1904] prepared *p*-bromophenylmercapturic acid in this way by condensing *p*-bromophenyldiazonium chloride with cysteine and acetylating the *p*-bromophenylcysteine so obtained. In our experiments α-naphthyldiazonium acetate was condensed with *d*-acetylcysteine and the condensation product decomposed in hot water. *l*-α-Naphthylmercapturic acid was obtained in poor yield and found to be identical with the mercapturic acid isolated from rabbits' urine.

Experimental.

d-Acetylcysteine was prepared by the method of Pirie and Hele [1933]. It melted at 108° (uncorr.) and a solution in water had $[\alpha]_D^{21°}$ 5·5° ($c=2·7$ %).

α-Naphthylamine (3·2 g.), recrystallised from light petroleum, was dissolved by warming in water (60 ml.) containing concentrated hydrochloric acid (2·5 ml.). The solution was cooled in ice with stirring and a further 2·5 ml. of concentrated hydrochloric acid and 30 g. of ice added. This suspension of α-naphthylamine hydrochloride was diazotised at 0° with a solution of sodium nitrite (1·6 g.) in water (15 ml.), which had been rendered acid to Congo paper by the addition of very dilute ice-cold hydrochloric acid [Bulow and Sprosser, 1908]. Sodium acetate (4 g.) in the least amount of water was then added to neutralise the hydrochloric acid and the solution filtered to remove a small amount of diazoaminonaphthalene. The filtrate was added slowly to a 10 % solution of *d*-acetylcysteine (3·2 g.) in water. An orange-coloured amorphous precipitate was formed, which was collected, suspended in water containing a few per cent. of alcohol and heated with the addition of copper-bronze powder on the water-bath until the evolution of nitrogen ceased. The hot solution was filtered, the

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copper powder and insoluble material washed with boiling water and the united filtrates evaporated to small bulk. The semi-crystalline solid material which separated was collected and dissolved in potassium carbonate solution, and the solution was filtered from resinous material and acidified. The crude mercapturic acid, which was precipitated, was extracted with chloroform, and the solid residue from the evaporation of the chloroform was crystallised by dissolution in a little alcohol and, after treatment with charcoal, addition of water. Yellowish crystals were obtained melting at 169–170°.

The crude acid (0·25 g. from 5 g. of acetylcysteine) was purified by conversion into its sodium salt which, like the sodium salt of the natural mercapturic acid, crystallises from excess of 2*N* sodium hydroxide. The free acid was recovered by acidifying an aqueous solution of the sodium salt and was crystallised from aqueous alcohol. This process was repeated once more and the acid finally crystallised from ethyl malonate, from which it separated in well-formed but still faintly coloured needles; yield 0·13 g. (Found: C, 62·30; H, 5·24; N, 4·60; S, 10·95 %. $C_{15}H_{15}O_3NS$ requires C, 62·28; H, 5·23; N, 4·84; S, 11·09 %.)

The synthetic α-naphthylmercapturic acid melted at 170–171°, the natural *l*-α-naphthylmercapturic acid at 170·5–171° and a mixture of the two at 170–171°.

The natural mercapturic acid in 1 % solution in alcohol had $[\alpha]_D^{25°} -25°$ and the synthetic acid in 1·005 % solution in alcohol had $[\alpha]_D^{25°} -23·9°$.

The synthetic *l*-α-naphthylmercapturic acid, like the natural product, was soluble in conc. H_2SO_4 and gave a green colour on warming.

Summary.

l-α-Naphthylmercapturic acid has been synthesised and proved to be identical with the mercapturic acid isolated from the urine of rabbits fed with naphthalene.

One of us (M. C. B.) is indebted to the Committee on the Physiology of Vision of the Medical Research Council for grants.

REFERENCES.

Bulow and Sprosser (1908). *Ber. deutsch. chem. Ges.* **41**, 1687.
Friedmann (1904). *Beitr. chem. Physiol.* **4**, 486.
Kalle and Co. (1907). *D.R.P.* 194040.
Pirie and Hele (1933). *Biochem. J.* **27**, 1716.

CXIV. A NOTE ON THE REDUCING ACTIVITY OF THE TISSUES OF NORMAL AND TUMOUR-BEARING RATS AND MICE.

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Harris and his colleagues have recently studied the reducing activity of acid extracts of a number of animal and vegetable tissues, and have found a correlation in most cases between this activity and the antiscorbutic potency of the tissues. Whether their technique [Birch, Harris and Ray, 1933] affords a specific test for such potency is still a matter of controversy but the authors themselves recognise the limitations of the method unless confirmed by biological assay. During the examination of the tissues Harris [1933, 1, 2] found that an extract of the Jensen rat sarcoma had a high reducing activity although he found by biological tests on guinea-pigs that this activity could not be attributed entirely to its ascorbic acid content. Similarly Borghi and Deotto [1934] were unable to demonstrate any antiscorbutic activity in extracts of the Ehrlich mouse carcinoma. Boyland [1933] confirmed the high content of reducing substances in the Jensen rat sarcoma but found a lower content for a second rat sarcoma, two mouse sarcomas, a mouse carcinoma (C 63) and a fowl sarcoma. In the case of the Jensen sarcoma but not in the other five tumours, the activity of the malignant tissues was higher than that of other tissues of the same animal. In commenting on Harris' results, Quastel [1933] cited in confirmation the greater reduction of ammonium molybdate by acid extracts of human tumours than by those of non-malignant tissues.

These observations have been extended in the present study to a number of different strains of transplantable rat and mouse tumours which have been propagated over a long period of years in the laboratories of the Imperial Cancer Research Fund. The reductions of both ammonium molybdate and 2:6-dichlorophenolindophenol by the tumours and other organs have been examined. The rats were maintained on a mixed diet of bread, meat biscuits, boiled beans, maize and kitchen scraps, and the mice on bread and oats, with cod-liver oil and yeast three times a week.

The reduction of ammonium molybdate by tissues of tumour-bearing rats and mice.

The following method was adopted for estimating the relative reducing activities of the tissues.

The tumour-bearing animal was killed and equal quantities of the different tissues ground up with dilute acetic acid and purified silver sand in hard glass centrifuge pots. 20 % ammonium molybdate was then added in equal amounts to each of the pots, which were incubated at 37° or 50° for 60 or 90 minutes.

The best results were usually obtained when 2 ml. of 3*N* acetic acid and 8 ml. of 20 % ammonium molybdate per 0·2 g. of tissue were used.

The intensity of the blue colour of the supernatant fluid after centrifuging was employed as a criterion of the degree of reduction of the molybdate.

Two rat tumours and eight mouse tumours were examined by this method and the results are shown in Table I.

Table I. *The reduction of ammonium molybdate by the tissues of rats and mice with transplantable tumours.*

	Tumour				Relative reducing values							
No.	Animal	Strain	Type	Age in days	Liver	Spleen	Brain	Muscle	Kidney	Lung	Necrotic tumour tissue	Non-necrotic tumour tissue
1	Rat	Jensen	Sarcoma	22	3	2	—	1	—	—	—	5
	,,	,,	,,	19	2	—	2	—	—	—	—	4
2	,,	Walker	Carcinoma	38	2	2	4	—	2	½	—	5
1	Mouse	Crocker	Sarcoma	15	2	½	—	0	—	—	—	4
2	,,	37	,,	14	2	⅓	—	1	—	—	—	4
3	,,	Bonne	Tar sarcoma	21	3	4	—	1	—	—	—	2
4	,,	173	,,	28	4	3	5	—	1	—	—	4
5	,,	2146	Tar carcinoma	20	2	4	—	1	—	—	—	4
	,,	2146	,,	15	1	2	—	½	—	—	—	3
6	,,	91	Carcinoma	66	5	4	—	1	1	—	—	4
7	,,	113	,,	28	2	3	—	½	1	—	½	4
8	,,	206	,,	18	2	3	4	—	—	—	1	—

The intensity of the blue colour was determined by comparison with an arbitrarily chosen standard, and the data in the right-hand columns afford an approximate comparison of the reducing activities of the tissues. The Jensen rat sarcoma and the Walker rat carcinoma showed activities of a higher order than the other tissues of the same animals. In the majority of the mouse tumours a similar increased activity of the malignant tissues was found. Exceptions were the Bonne and the 173 sarcomas and the 91 carcinoma, where the spleen, brain and liver respectively showed greater reducing activities. A slight reduction of the ammonium molybdate was effected even by the highly necrotic portions of a tumour as was shown in the case of tumours 113 and 206. Both the spleen and brain showed high reducing activities and in most cases these organs were superior to the liver in this respect.

The reduction of 2:6-dichlorophenolindophenol by acid extracts of the tissues of normal and tumour-bearing rats and mice.

A slight modification of the technique of Birch *et al.* [1933] was employed for testing the reducing activity of the extracts.

The animals were killed and the organs removed. 2 ml. of 10 % ice-cold trichloroacetic acid were added to small weighed quantities of each of the different organs which were then ground with 0·5 g. of purified silver sand in small hard glass centrifuge-pots. 2 ml. of cold distilled water were added in each case, and the material was centrifuged for 5 minutes. After centrifuging, the supernatant acid extracts were poured off into labelled beakers. 4 ml. of distilled water were added to the mixture of sand and tissue in each case and the centrifuging was continued for a further 5 minutes. This second supernatant was then added to the contents of the corresponding beaker. A third washing was found to be unnecessary. The 8 ml. of extract and washings contained therefore the acid extractives of the original weighed tissue.

These extracts were then titrated against a measured volume of the 2:6-dichlorophenolindophenol indicator. The indicator solution was made by dissolving 0·05 g. of the dried substance in a small quantity of boiling distilled water and filtering. The undissolved residue was extracted with a further quantity of boiling water and the total volume of the filtrate made up to 50 ml. The tissue extracts under examination were run from a micro-burette into 1·0 or 0·5 ml. of a 1 in 10 dilution of the indicator as prepared above, in a small beaker. Table II shows the reducing activities of the various tissues. The figures represent the ml. of the indicator solution (1 ml. of which was equivalent to 0·04 mg. of a commercial sample of ascorbic acid) reduced by the extract from 1 g. of tissue.

Table II. *The reducing activity of the acid extracts of the tissues of normal and tumour-bearing rats and mice.*

		Tumour			Relative reducing activities of tissues ml. 2:6-dichlorophenolindophenol solution (1/10,000) reduced by extract from 1 g. of tissue								
No.	Animal	Strain	Type	Age in days	Liver	Spleen	Brain	Kidney	Lung	Testes	Embryo	Necrotic tumour tissue	Non-necrotic tumour tissue
1	Rat	Jensen	Sarcoma	31	5·2	8·7	8·7	3·5	—	—	—	—	10·7
	,,	,,	,,	8	8·2	8·8	7·8	5·7	—	—	—	—	11·2
	,,	,,	,,	26	—	—	8·3	—	—	—	—	—	10·8
2	,,	Walker	Carc.	39	7·6	9·4	9·7	—	8·4	—	—	6·0	14·5
	,,	,,	,,	80	4·9	7·5	6·6	3·0	—	—	—	—	13·3
	,,	,,	,,	21	3·8	6·9	4·9	3·1	5·2	—	—	—	10·7
	,,	Normal	—	—	10·0	8·7	8·0	5·3	5·7	8·0	—	—	—
	,,	,,	—	—	10·0	9·7	—	5·8	8·2	7·4	—	—	—
	,,	,,	—	—	9·1	9·1	8·6	5·7	3·7	8·4	—	—	—
1	Mouse	Crocker	Sarcoma	21	10·5	14·5	14·3	9·0	13·3	—	—	—	14·1
2	,,	37	,,	13	9·5	8·3	16·3	5·0	8·0	—	—	—	7·2
	,,	37	,,	25	8·4	8·2	12·3	7·5	—	—	—	—	9·2
3	,,	Bonne	Tar sarc.	7	7·3	—	11·6	—	—	—	—	—	10·0
4	,,	173	,,	15	8·5	7·6	13·1	—	8·5	—	8·0	—	6·7
	,,	173	,,	22	5·7	10·0	12·5	—	6·9	—	—	—	6·7
5	,,	2146	Tar carc.	9	10·5	11·2	11·1	—	—	—	—	—	9·5
6	,,	113	Carc.	80	8·3	13·0	14·0	—	12·0	—	—	—	14·3
7	,,	4108	Sporadic	—	9·5	16·3	13·5	8·0	—	—	—	11·9	16·0
	,,	Normal	—	—	12·3	16·0	15·7	9·6	15·8	—	—	—	—
	,,	,,	—	—	8·9	10·4	14·8	7·0	14·0	—	—	—	—
	,,	,,	—	—	9·0	12·7	17·0	7·6	9·3	—	—	—	—

Both the Jensen and Walker tumours showed greater activity than the other tissues of the same animals. The variation in the reducing values of the rat tumours was small (minimum 10·7; maximum 14·5). There was a greater variation in the reducing values of the different strains of mouse tumours (minimum 6·7; maximum 16·0) and in most cases the values were of a lower order than those for the spleen or brain. The high reducing activity of the brain tissue is of interest in connection with some experiments which one of us (M. M.) has carried out in collaboration with Dr F. G. Young in the Biochemistry Department at University College, London [Young and Mitolo, 1934]. The general properties of the reducing substance prepared from ox brain clearly differentiate it from ascorbic acid and in this respect it shows a similarity to the reducing substance in tumour tissue [Harris, 1933, 2; Borghi and Deotto, 1934].

Tumour 4108 was the 6-weeks old autoplast of a spontaneously occurring mammary carcinoma. Both the non-necrotic and the necrotic portions had high reducing values. The values for the tissue extracts of normal tumour-free rats and mice were of a similar order to those of the tumour-bearing animals.

Although there is a considerable variation in the reducing values of the transplantable tumours of different strains, the values of tumours of the same strain show less variation even under different conditions. This is shown by the similarity of the values for (*a*) tumours of the same or different ages, (*b*) multiple transplantable tumours of the same animal, and (*c*) progressing and spontaneously regressing transplantable rat tumours.

Tumours of the same and different ages. In a series of nine 2146 mouse carcinomas the reducing values were 10·0, 9·0, 8·7, 8·5, 9·5, 11·0, 10·2, 9·4 and 9·5 after the same period of growth and before the tumours showed any necrosis. The values for the Jensen and Walker rat tumours and the mouse tumours 37 and 173 of different ages were very similar (Table II).

Multiple transplantable tumours. In cases where two transplants were made in the same animal the reducing values of the subsequent tumours were approximately the same, *e.g.*:

	Relative reducing activities	
	Left tumour	Right tumour
2146 (mouse tar carcinoma) 1.	9·5	9·2
2.	10·2	10·0

Progressing and spontaneously regressing tumours. In two cases Jensen rat tumours which were showing rapid spontaneous regression were found to have reducing values of 10·9 and 12·5. These values were very similar to those found for the normal progressing tumours (10·7, 11·2, 10·8, Table II).

Summary.

1. Transplantable rat and mouse tumours of different strains readily reduce ammonium molybdate in an acid medium. In most cases the tumours show a higher content of reducing substances than the liver, spleen or brain.

2. The reducing substances in these tumours are soluble in dilute trichloroacetic acid, and extracts obtained in this way reduce 2:6-dichlorophenolindophenol. In the case of the rat strains the tumour extracts were more active than those of the liver, spleen or brain. They were somewhat less active than these extracts however in the case of the mouse strains.

3. Although there is a considerable variation in the reducing activities of the tumours of different strains, the values of the tumours of the same strain are comparatively constant. This is shown by the similarity of the reducing values for (*a*) tumours of the same and different ages, (*b*) multiple transplantable tumours in the same animal, and (*c*) progressing and spontaneously regressing tumours.

We are indebted to our colleague Dr Cramer for a number of the tumours used in these studies and to Mr H. E. Adams for help with the analyses.

REFERENCES.

Birch, Harris and Ray (1933). *Biochem. J.* **27**, 590.
Borghi and Deotto (1934). *Z. Krebsforsch.* **40**, 293.
Boyland (1933). *Biochem. J.* **27**, 802.
Harris (1933, 1). *Nature*, **132**, 27.
—— (1933, 2). *Nature*, **132**, 605.
Quastel (1933). *Nature*, **132**, 101.
Young and Mitolo (1934). *Nature*, **133**, 572.

CXV. THE FORMATION OF COPROSTEROL IN THE INTESTINE[1].

I. POSSIBLE RÔLE OF DIHYDROCHOLESTEROL, AND A METHOD OF DETERMINING DIHYDROCHOLESTEROL IN PRESENCE OF COPROSTEROL.

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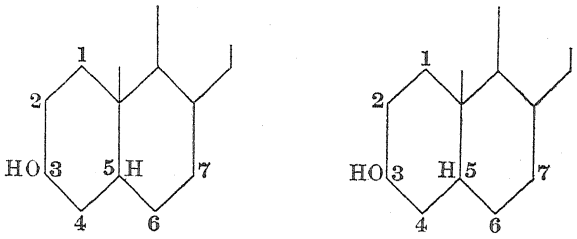
Cholesterol of exogenous or endogenous origin is eliminated with the faeces mainly in the form of coprosterol. Soon after the discovery of this compound by Bondzynski and Humnicki [1896–97] it was suggested that coprosterol is formed by bacterial hydrogenation of cholesterol in the intestine, but no definite proof of this theory has as yet been forthcoming. All attempts to convert cholesterol into coprosterol by bacteria *in vitro* have been unsuccessful.

Human faeces contain, besides coprosterol, small quantities of unchanged cholesterol and dihydrocholesterol [Windaus and Uibrig, 1915]. Schönheimer *et al.* [1930] have shown that dihydrocholesterol is formed from cholesterol in the body tissues (or synthesised there) and is continuously secreted into the intestine, but intensive search for coprosterol in the organs has failed to reveal it even in traces. It is possible that the intestinal bacteria effect the stereochemical inversion of the secreted dihydrocholesterol at carbon atom 5 rather than the hydrogenation of the double bond in cholesterol. Were this the case, ingested dihydrocholesterol should, at least in part, reappear as coprosterol



Dihydrocholesterol (abbreviated) Coprosterol

in the faeces. Feeding experiments designed to test this view can be carried out approximately quantitatively because neither coprosterol nor dihydrocholesterol can be absorbed from the intestine [von Behring and Schönheimer, 1930].

To this end a method for the quantitative determination of dihydrocholesterol in presence of coprosterol is necessary. As these two isomerides closely

[1] This work was aided by a grant from the Josiah P. Macy, Jr., Foundation.

resemble each other in physical and chemical properties, few ways of doing this offer themselves. At first an attempt was made to estimate the amount of both sterols in a mixture with the aid of the fact that they are converted by sodium ethylate (or sodium in xylene) into the non-precipitable isomerides (epicholestanol and epicoprosterol) to a different extent. This method proved to be impracticable for analytical purposes as the values were not well reproducible. The method finally adopted involves the differential solubility of the digitonides of the two substances [Schönheimer and Dam, 1933]; coprosterol digitonide dissolves to the extent of 2 g. in 100 ml. of methyl alcohol, while the corresponding solubility of dihydrocholesterol digitonide is only 0·35 g. A curve can, therefore, be constructed whereby the composition of a mixture of the two can be determined directly from its solubility in methyl alcohol.

With the aid of this method it has been possible to show that dihydrocholesterol given by mouth is excreted unchanged and is not converted into coprosterol.

Experimental.

1. *Curves for the solubility of mixtures of the two sterol digitonides in methyl alcohol.*

The digitonides were prepared by precipitation of 0·5 % alcoholic solutions of the sterol mixtures with digitonin (50 % excess, time of precipitation 24 hours). On account of the solubility of coprosterol digitonide in ethyl alcohol, the precipitates were washed with 20 ml. of a saturated solution of coprosterol digitonide in 96 % alcohol and finally with 1 ml. of ether. For the solubility determination, quantities of about 135 mg. of the digitonide (dried at 110°) were weighed out in small flat-bottomed glass vessels and again dried; a loss of a few mg. was observed owing to the hygroscopic character of the substance. Then enough of the product was taken out to leave $127 \pm 0{\cdot}5$ mg. in the vessel, which was placed in a narrow, stoppered glass cylinder and covered with a rubber cap, and the cylinder shaken overnight in a thermostat at $25 \pm 0{\cdot}1°$. The solution was then filtered and the dry residue determined in 2 ml. of the filtrate. Great care must be taken that the methyl alcohol is dry, for the least trace of moisture depresses the solubility of the digitonide. The methyl alcohol for these experiments was dried by boiling for 20 minutes with calcium turnings and distilled with the exclusion of moisture by means of a calcium chloride tube. Extreme precautions were also taken to avoid contamination by moisture during storage.

Since the separation of the saturated sterols from cholesterol by the method of Schönheimer involves preliminary treatment with bromine, a solubility curve was also constructed of the digitonides which had been precipitated in alcohol containing sufficient bromine to give it a yellow colour. The presence of bromine slightly raised the solubility of the digitonides (curve 2, Fig. 1). All the values were obtained with the same digitonin preparation, for digitonins of different sources are known to possess slightly different properties.

An attempt was made by this method to estimate dihydrocholesterol added to the mixture of saturated sterols isolated from faeces. The saturated sterols from the faeces of a human subject whose diet contained very little plant sterol were isolated after bromination by the method of Schönheimer [1930]. The resulting digitonide had a solubility of 43·8 mg. in 2 ml. Since pure coprosterol digitonide has a solubility of 41·7 mg. (when precipitated in presence of bromine) this finding indicates that traces of an unknown more soluble substance were present in the mixture. Attempts to isolate a more soluble saturated fraction

by fractional precipitation with digitonin of the mother-liquor from the preparation of coprosterol led only to a digitonide with a maximum solubility of 46·7 mg. in 2 ml. of methyl alcohol. In the feeding experiments a special curve (No. 3 in Fig. 1) was therefore employed which related not to the content of dihydrocholesterol in a mixture with pure coprosterol but to the composition of mixtures of dihydrocholesterol and the saturated sterol of faecal origin.

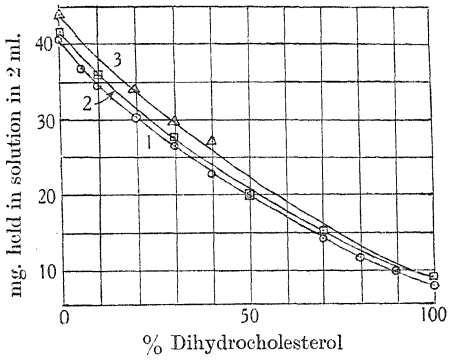


Fig. 1. Solubility of digitonides from coprosterol and dihydrocholesterol in absolute methyl alcohol at 25°, 127 mg. digitonide + 5 ml. methyl alcohol. 1. Digitonides precipitated without bromine. 2. Digitonides precipitated in presence of bromine. 3. Digitonide of sterol from human faeces + dihydrocholesterol, precipitated in presence of bromine.

The experimental findings are reproduced in Fig. 1. With the aid of these curves good recovery of added dihydrocholesterol was made from both coprosterol and the sterols of faeces. By means of curve 1 it was found that on hydrogenation of 0·2 g. allocholesterol with 0·2 g. of platinum oxide with hydrogen in cold ether, 55 % of coprosterol and 45 % of dihydrocholesterol were produced. The solubility of the digitonides of the reaction product was 23·8 mg. in 2 ml. of methyl alcohol.

2. *Feeding experiments with dihydrocholesterol.*

A healthy individual was kept on a constant diet of which the sterols were almost entirely of animal origin. The diet for each period consisted of 930 g. butter, 400 g. 30 % cream, 280 g. lean pork, 500 g. potatoes, 100 g. barley porridge, 732 g. zwieback and 40 g. Graham bread. During the experimental period he received a supplement of dihydrocholesterol approximately corresponding to the amount of total sterols excreted in the faeces. The total and saturated sterols were determined in the stools during a pre-experimental, an experimental and two post-experimental periods, and the solubility of the saturated sterol digitonides was measured. Each period occupied 5 days, of which the last was made a fast day in order to obtain a better differentiation of the periods (the day before the beginning of the experiment was also a fast day). The beginning of each period in the faeces was marked by a carmine tablet.

During the experimental period 3·45 g. of dihydrocholesterol, dissolved in butter, were taken, distributed through 12 meals. Immediately after defaecation the stools were ground with sodium sulphate and finely powdered, an accurately weighed sample (corresponding roughly to a 24-hour quantity) was extracted with ether and the amounts of total sterols and saturated sterols determined by Schönheimer's method. The precipitation of the digitonide of

the saturated sterols, employing 50 % excess of digitonin, was carried out after bromination in alcoholic solution. The solubility of the precipitates was determined, and the content of dihydrocholesterol calculated from curve 3, Fig. 1.

Table I. *Balance experiments with dihydrocholesterol.*

	Pre-experimental period	Experimental period 3·45 g. added dihydrocholesterol	1st post-experimental period	2nd post-experimental period
	g.	g.	g.	g.
Total unsaponifiable	5·35	9·91	7·22	5·71
Total sterol	4·23	8·42	5·88	4·26
Saturated sterol	3·65	7·41	5·20	3·42
Solubility of digitonides of saturated sterols in 2 ml. methyl alcohol:				
	mg.	mg.	mg.	mg.
Determination A	43·8	27·1	33·4	44·3
Determination B	43·9	27·6	35·0	43·6
Amount of dihydrocholesterol:				
Determination A	0 %	38 %	20 %	0 %
Absolute amount	0 g.	2·81 g.	1·04 g.	0 g.
Determination B	0 %	37 %	18 %	0 %
Absolute amount	0 g.	2·74 g.	0·94 g.	0 g.

From Table I it will be seen that dihydrocholesterol added to the diet was not excreted entirely during the experimental period, but a considerable portion appeared during the first post-experimental period. The whole amount of ingested dihydrocholesterol was recovered within these two periods. 3·85 g. dihydrocholesterol were found in the first determination and 3·68 g. in the second. The limit of error in the rather complicated experiment must however be considered relatively high, perhaps 10–20 %.

Under the influence of the relatively large quantities of ingested dihydrocholesterol the resorption of part of the cholesterol is apparently prevented, with resulting bacterial conversion into coprosterol (see the following paper), for during the pre-experimental period and the second post-experimental period 7·07 g. in all of saturated sterols were excreted. During the experimental and first post-experimental periods a total of 12·61 g. of saturated sterol was found, an increase of 5·54 g. Since 3·68–3·85 g. of this were found to consist of dihydrocholesterol, the missing 2 g. must have consisted of coprosterol. This cannot have been formed from the dihydrocholesterol inasmuch as the latter was recovered practically quantitatively.

The inhibition of the resorption of cholesterol by the ingestion of dihydrocholesterol can be explained on physical grounds, for cholesterol and dihydrocholesterol are known to form mixed crystals which cannot be separated by physical methods [Schönheimer, 1930: Lettré, 1932].

The amount of non-sterol unsaponifiable matter excreted in the experimental and first post-experimental periods is increased only to a small extent (10 %) over the corresponding excretion during the experimental and first post-experimental periods. This is barely beyond the limit of variation and thus supports the view that the non-sterol unsaponifiable matter does not originate from unresorbed cholesterol to more than a small extent.

SUMMARY.

A method is described for the determination of dihydrocholesterol in presence of coprosterol depending on the differential solubility of the corresponding digitonides in methyl alcohol.

With its aid it is shown that dihydrocholesterol added to the diet is not converted into coprosterol but reappears unchanged in the faeces.

The author wishes to express his indebtedness to Prof. R. Schönheimer, formerly Head of the Department of Chemistry in the Pathological Institute, Freiburg, for many suggestions during the progress of this work.

REFERENCES.

von Behring and Schönheimer (1930). *Z. physiol. Chem.* **192**, 97.
Bondzynski and Humnicki (1896–97). *Z. physiol. Chem.* **22**, 396.
Lettré (1932). *Liebig's Ann.* **495**, 41.
Schönheimer (1930). *Z. physiol. Chem.* **192**, 77.
—— von Behring and Hummel (1930). *Z. physiol. Chem.* **192**, 73.
—— and von Behring (1930). *Z. physiol. Chem.* **192**, 102.
—— and Dam (1933). *Z. physiol. Chem.* **215**, 59.
Windaus and Uibrig (1915). *Ber. deutsch. chem. Ges.* **48**, 857.

CXVI. THE FORMATION OF COPROSTEROL IN THE INTESTINE[1].

II. THE ACTION OF INTESTINAL BACTERIA ON CHOLESTEROL.

By HENRIK DAM.

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ALTHOUGH various investigators [Bondzynski and Humnicki, 1896–97; Schönheimer *et al.*, 1930; Beumer and Bischoff, 1930] had reported the failure of attempts to convert cholesterol into coprosterol by bacteria *in vitro*, it was stated by Bischoff [1930, 1, 2] that the degree of saturation of faecal sterols increases when faeces are putrefied. This result, obtained by applying the iodine method of Bürger and Winterseel [1929] to sterols isolated from their digitonides by extraction with xylene, points to the probability that coprosterol can be formed by bacterial action.

An easier method is now available for the determination of total saturated sterols in the presence of unsaturated sterols [Schönheimer, 1930], and a method for the determination of dihydrocholesterol and coprosterol in a mixture has been described in the preceding paper. With the aid of these methods I have been able to confirm Bischoff's statement and to show that under the conditions of intestinal putrefaction cholesterol is converted not into dihydrocholesterol but into coprosterol.

Most of the putrefaction experiments were conducted quantitatively, and it was found that the total sterol was constant during putrefaction, indicating that intestinal bacteria are not able to destroy cholesterol or coprosterol.

EXPERIMENTAL.

The putrefaction experiments were generally carried out in an atmosphere of nitrogen or hydrogen although some were performed under aerobic conditions. When the putrefaction was conducted in the absence of oxygen, water or mercury seals were employed; under aerobic conditions the vessels were closed merely with a cotton plug. (In the aerobic experiments the processes in the inner mass of the faeces were most probably anaerobic because of the slow diffusion of gases from the surface.)

It was noted that in putrefaction under hydrogen the gas disappeared in amounts greatly exceeding those corresponding to the hydrogenation of the cholesterol.

The faeces were rubbed up to a uniform consistency in a mortar and weighed out with an accuracy of about 50 mg. Unless otherwise stated in the tables, 10 g. of faeces were employed in each experiment. The unsaponifiable matter

[1] This investigation was made possible by the assistance of the Josiah Macy, Jr., Foundation.

was isolated, and the saturated sterols were determined according to the method of Schönheimer [1930]. Only when the degree of saturation was very small (when cholesterol was added to the faeces or with a milk diet) did cholesterol dibromide crystallise from the alcohol on addition of bromine. In general 30–100 mg. of digitonide were obtained; only with low degrees of saturation were the amounts smaller than this, in which case the accuracy of the results was correspondingly influenced.

1. *Putrefaction of faeces without additions.*

Table I.

		Total sterols in 10 g.				
		Before incubation		After incubation		
	Diet	Amount mg.	Saturation %	Amount mg.	Saturation %	Incubation
1. Adult (H. D.)	Mixed	94·8	91·6	92	92·6	Aerobic, 49 hrs., 40°
2. ,, (S)	,,	63·0	78·6	65	88	In H_2, 7 days, 38°
3. ,, (corpse)*	,,	75·2	80·0	77·8	87·5	In H_2, 10 days, 38°
4. ,, (E. J.)	Milk†	59·8	69·6	61·8	79·5	In H_2, 48 hrs., 37°
5. ,, (I. D.)	Milk‡	85·2	2·5	85·3	1·8	In H_2, 67 hrs., 38·5°
6. Infant (8 days old)	Milk	81·2	8·5	80·2	9·1	Aerobic, 48 hrs., 37°

* Colon contents.
† 4 litres daily, beginning 4 days before experiment.
‡ 1 litre milk, 0·5 litre 30 % cream, 1 zwieback, daily, beginning 4 days before experiment.
p_H of the faeces = 8·1 (quinhydrone) (a few days earlier the faeces were acid).

In none of the experiments was a decrease observed in the total sterols during putrefaction, the difference lying within the limits of error. In Exps. 2, 3 and 4 hydrogenation of the sterols is clearly evident. No hydrogenation occurred in Exp. 1, which may be ascribed to the fact that the initial degree of saturation was very high (92 %). These experiments confirm the observation of Müller [1900] that the degree of saturation of the sterols falls on a milk diet. In cases of extremely low initial saturation (Exps. 5 and 6) no hydrogenation occurs during putrefaction.

2. *Putrefaction of the contents of the jejunum and ileum.*

The amount of cholesterol hydrogenated during putrefaction of colon contents and faeces even in the positive experiments could be only small, for the major part of the sterols existed at the outset in the form of coprosterol. An experiment (No. 7, not reported in the Tables) was accordingly undertaken with the united contents of the jejunum and ileum, in which hydrogenation has, as a rule, proceeded only to a slight extent. The material employed was obtained from the individual whose colon contents were examined in Exp. 3 (Table I). On analysis 10 g. of the fresh material showed 23·6 mg. of total sterols of which 2·5 % was saturated; after incubation for 10 days at 38° in H_2 the total sterols were 24·8 mg. with 2·6 % saturation. The hydrogenation of sterols thus appears to be associated with the contents of the large intestine.

3. *Putrefaction of faeces with added cholesterol.*

As the cholesterol for these experiments had to be in a form that could be attacked by bacteria, crystalline material was unsuitable. Egg-yolk, known to be rich in cholesterol, was employed; or cholesterol was either brought into

solution in bile acids and fatty acids or employed in the form of the water-soluble ester salts synthesised by Schönheimer and Breusch [1932]. In later experiments (Tables III and IV) colloidal cholesterol solutions secured from the firm of von Heyden were also employed. All added solutions were neutralised to litmus.

Table II.

		Substance	State	Total sterols in 10 g. Amount mg.	Total sterols in 10 g. Saturation %	Incubation
8.	Adult (H. D.)	35 g. faeces + 15 g. egg-yolk	Fresh	120	61·4	—
			Incub.	120	64·9	Aerobic, 48 hrs., 37°
9.	,, (E. J.)	100 g. faeces + 18 g. egg-yolk	Fresh	93·4	69·3	—
			Incub.	93·5	85·4	In H_2, 49 hrs., 39–43°
10.	,, (S)	8 g. faeces + 10 ml. sol. A	Fresh	—	33·2	—
			Incub.	—	36·1	In H_2, 4 hrs., 37°
			,,	—	34·4	In H_2, 47 hrs., 37°
11.	,, (corpse)	60 g. colon content + sol. B	Fresh	207	60·7	—
			Incub.	204·5	74·7	In H_2, 18 days, 37°
12.	,, (S)	8 g. faeces + 1·5 ml. sol. C	Fresh	115·2	43·8	—
			Incub.	114	50·8	In H_2, 7 days, 38°
			,,	117	52·3	In N_2, 7 days, 38°
			,,	113	43·8	Sterilised in boiling water-bath, then 7 days at 38° in H_2
13.	,, (S)	20 g. faeces + 3 ml. sol. D	Fresh	—	45	—
			Incub.	—	58·4	In H_2, 9 days, 37°

All were on mixed diet.
Solution A. 6·2 g. sodium desoxycholate, 1 g. sodium stearate and 0·35 g. cholesterol in 30 ml. water.
,, B. 1·2 g. sodium salt of monocholesterylbutanetetracarboxylic acid in 8 ml. water. The whole solution was added.
,, C. 1·5 g. sodium salt of monocholesterylbutanetetracarboxylic acid in 25 ml. water.
,, D. 0·6 g. sodium salt of monoallocholesterylbutanetetracarboxylic acid in 6·5 ml. water.

Cholesterol in egg-yolk or in the form of soluble preparations was only slightly hydrogenated, the increase in saturated sterols being little more than that obtained without the addition of cholesterol.

A parallel experiment in which allocholesterol was added in the form of its soluble acid ester (Exp. 13) was also carried out. The failure of an attempt to convert allocholesterol into coprosterol by putrefaction has been reported by Beumer and Bischoff [1930]; these authors however had heated the allocholesterol before the experiment, a procedure which may have converted it into cholesterol. The soluble allocholesterol ester was not converted any more rapidly than the analogous cholesterol ester. The addition of large quantities of bile acids and fatty acids appears to diminish the extent of hydrogenation.

4. *Effect of formates on putrefaction.*

Sodium formate, which is an efficient hydrogen donator for *B. coli* and other bacteria, was without influence.

In Exp. 14 the rather extensive hydrogenation was not increased by formate. Essentially the same result was obtained in Exp. 15, in which it was found that cholesterol betaine ester was practically not hydrogenated.

Table III.

	Substance	State	Saturation of sterols %	Incubation
14.	9 g. faeces + colloidal cholesterol solution	Fresh	62·7	—
		Incub.	96·5	In N_2, 6 days, 38°
		,,	92·5	Ditto, with 3 ml. 5 % H.COONa
15.	3 g. faeces + 86 mg. cholesterylbetaine ester hydrochloride	Fresh	22·9	—
		Incub.	23·5	Aerobic, 12 days, 38°
		,,	27·7	Ditto, with 3 ml. 5 % H.COONa

Subjects were adults on mixed diet.

5. *Attempted hydrogenation with pure bacterial cultures*[1].

Table IV.

		Saturation of sterols %
16.	Human bile (gall bladder), 170 ml. + 17 ml. 5 % H.COONa + 4·9 g. *B. coli*. Incubated aerobically for 10 days	4·0
17.	Brain hash (human) 62 g. + 6·2 ml. 5 % H.COONa inoculated with *B. coli*. Incubated *in vacuo* 5 days	1·9
18.	Brain hash (human) 37 g. + 3·6 ml. 5 % H.COONa, inoculated with *B. coli*. Incubated *in vacuo* 5 days	0·3
19.	Colloidal cholesterol in protein (von Heyden) 1 g. + 4 ml. water + 700 mg. *B. coli*. Incubated *in vacuo* 12 days	5·7
20.	Colloidal cholesterol in protein, 1 g. + 5 ml. 5 % H.COONa + 1·16 g. *B. coli*. Incubated *in vacuo* 12 days	0·6
21.	Colloidal cholesterol solution (von Heyden), 5 ml. + 20 ml. nutrient bouillon with 10 % glucose, inoculated with *Enterococcus* Type B. Incubated aerobically 2 days	*

* The amount was too small to weigh.

The action of pure bacterial cultures was, in spite of the large amounts of bacteria employed, barely perceptible, and certainly considerably less than in putrefaction of faeces.

6. *Nature of the saturated sterols formed during putrefaction.*

The digitonin-precipitable, saturated sterols formed from cholesterol can consist only of coprosterol or dihydrocholesterol, so that the isolation of the saturated sterol formed by putrefaction cannot be accomplished by methods at present available. In the previous communication however a method has been described for determining which of the two sterols has been formed. Since relatively large quantities of sterol are necessary for the solubility determinations, the digitonides from Exps. 2 and 12 were employed. Both experiments involved the same sample of stool, of which a large quantity was available. In Exp. 12 soluble cholesterol ester was added. The appropriate combined digitonides from Exps. 2 and 12 were united for the determination of sterols before and after putrefaction, respectively. During putrefaction 11–13 % of saturated sterols had formed; if these had consisted of dihydrocholesterol, the solubility of the digitonides in methyl alcohol would have been considerably lower than before putrefaction, whereas if coprosterol had formed, the solubility would barely have changed. Actually the solubilities were 42·0 and 43·4 mg. in 2 ml.

[1] Thanks are due to Miss R. Marget for valuable help in the bacteriological work.

of methyl alcohol respectively, before and after putrefaction. If dihydrocholesterol had been the sole product of hydrogenation, the solubility of the sterol-digitonide after putrefaction would have been about 37 mg.

Discussion.

The observation that cholesterol and coprosterol are not degraded by intestinal bacteria is of importance in the interpretation of cholesterol balance experiments. It is therefore possible to ascribe the destruction of cholesterol in the body [Dam, 1931; Page and Menschick, 1932; Menschick and Page, 1933; Schönheimer and Breusch, 1933] to a process of intermediary metabolism rather than to the agency of intestinal bacteria.

The present results confirm those of Bischoff [1930, 1, 2] in indicating that the amount of saturated sterols increases, but they differ from them quantitatively. In one case Bischoff found an increase from 12 to 66 %; I have never observed so large a change. The values found by Bischoff for the degree of saturation of the sterols of normal human faeces are rather low in comparison with the observations of Schönheimer and von Behring [1930] and those here reported which were found by the gravimetric method of Schönheimer. This discrepancy may perhaps be explained by some technical error in the determinations of Bischoff—an interpretation supported by the statement of Bischoff that a coprosterol with a melting-point of 97° employed by him was precipitable by digitonin only to the extent of 60 %.

Although hydrogenation took place only to a small extent on putrefaction, its occurrence was conclusively established in almost every experiment with normal faeces. It failed to occur only when the initial degree of saturation was extremely high (Exp. 1), and in some of the milk diet experiments in which the initial saturation was extremely low. Complete hydrogenation of unsaturated sterol was never attained, even on long incubation.

Added cholesterol, whether in the form of egg-yolk, as soluble ester or in colloidal solution, remained largely unattacked by the bacteria. It thus seems possible that the cholesterol exists in the colon in some unknown form which is especially susceptible to bacterial action.

The hydrogenation is certainly a bacterial process. It does not take place after sterilisation (Exp. 12), and it is associated particularly with the contents of the colon, for it takes place neither in the small intestine nor on the inoculation of cholesterol-containing organs or soluble cholesterol preparations with bacterial cultures. It may be supposed that either the optimum conditions for the growth of the appropriate bacteria can be obtained only in the colonic secretions, or these secretions contain special hydrogen donators. Sodium formate can apparently not be employed by the bacteria for the formation of coprosterol. Better results might perhaps be obtained by the use of sterile colon excretions as nutrient medium, but such material was not available.

The identity of the causative bacteria could not be determined.

The proof, by solubility determinations of the digitonides, that the hydrogenated sterol is not dihydrocholesterol but coprosterol establishes the fact, long suspected, that it is coprosterol which is formed by bacterial hydrogenation of cholesterol.

Summary.

In the putrefaction of faeces the degree of saturation of the sterols rises owing to formation of coprosterol from cholesterol. The total amount of sterol remains constant.

The author wishes to acknowledge his indebtedness to Prof. R. Schönheimer, formerly Head of the Department of Chemistry, Institute of Pathology, Freiburg, for many helpful suggestions.

REFERENCES.

Beumer and Bischoff (1930). *Biochem. Z.* **220**, 154.
Bischoff (1930, 1). *Biochem. Z.* **222**, 211.
—— (1930, 2). *Biochem. Z.* **227**, 230.
Bondzynski and Humnicki (1896–97). *Z. physiol. Chem.* **22**, 396.
Bürger and Winterseel (1929). *Z. exp. Med.* **66**, 471.
Dam (1931). *Biochem. Z.* **232**, 269.
Menschick and Page (1933). *Z. physiol. Chem.* **218**, 95.
Müller (1900). *Z. physiol. Chem.* **29**, 129.
Page and Menschick (1932). *J. Biol. Chem.* **97**, 359.
Schönheimer (1930). *Z. physiol. Chem.* **192**, 77.
—— von Behring, Hummel and Schindel (1930). *Z. physiol. Chem.* **192**, 74.
—— and von Behring (1930). *Z. physiol. Chem.* **192**, 102.
—— and Breusch (1932). *Z. physiol. Chem.* **211**, 19.
—— —— (1933). *J. Biol. Chem.* **103**, 439.

CXVII. THE MELTING-POINT AS A TEST FOR THE PURITY OF COPROSTEROL[1].

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RECENT work on the metabolism of saturated sterols makes it necessary to know the reliability of the usual tests for the purity of coprosterol.

The degree of saturation can be easily determined by the bromine-digitonin method of Schönheimer [1930] with an accuracy of about 1 %, so that it is possible to detect cholesterol as an impurity in the preparation. This method can also be used for the removal of any cholesterol, thus rendering it possible to obtain a preparation with a saturation of nearly 100 %, even in cases where the crude product contains a large amount of unsaturated sterols.

As shown by Windaus and Uibrig [1915], Gardner [1921] and Schönheimer [1930], dihydrocholesterol occurs in the sterol fraction of faeces and may therefore be found as an impurity in coprosterol prepared from this source.

The specific rotations for coprosterol and dihydrocholesterol are $[\alpha]_D$ +23·55° [Windaus, 1916] and +28·8° [Willstätter and Mayer, 1908] respectively, and are thus too close for use in the simultaneous determination of these two sterols.

The melting-point of coprosterol is variously given as 95–96° [Bondzynski and Humnicki, 1896–97], 100° [Dorée, 1913], 112–116° [Fischer, 1911], 101–102° [Windaus, 1916]; a product prepared from pseudocoprosterol melted at 105–107° [Uibrig, 1915]. The highest recorded (Fischer) was on a sample many times recrystallised, but subsequently [Windaus and Uibrig, 1915] shown nevertheless to contain dihydrocholesterol. An equally high melting-point has also been reported by Schönheimer [1929] and by the present author [1925].

In order to ascertain the depression of the melting-point of coprosterol by either dihydrocholesterol or cholesterol, the melting-point diagram has been determined by the method of Rheinboldt and Kircheisen [1926] and Lettré [1932].

The material used was prepared from human faeces collected on a diet (white bread, butter, meat, eggs, potatoes and barley porridge) poor in plant sterols. The digitonide of the unsaponifiable fraction was decomposed by extraction with xylene and the sterol recrystallised from methyl alcohol. It melted at 100–101°, unchanged after repeated crystallisation. The degree of saturation (method of Schönheimer) was 98·7 %. Only a faint yellow colour was obtained in the Salkowski reaction.

The melting-point diagram shows that the presence of up to about 50 % of dihydrocholesterol will produce only a slight depression of the melting-point of the coprosterol. The softening-points of the mixtures were materially lower, but very difficult to determine.

[1] This investigation was made possible by a grant from the Josiah Macy, Jr., Foundation.

Fig. 1 shows that in the neighbourhood of 100° the sharpness of the melting-point is of much greater importance as a test for purity than the melting-point itself. A melting-point of 110–115° represents a mixture with more than 60 % dihydrocholesterol.

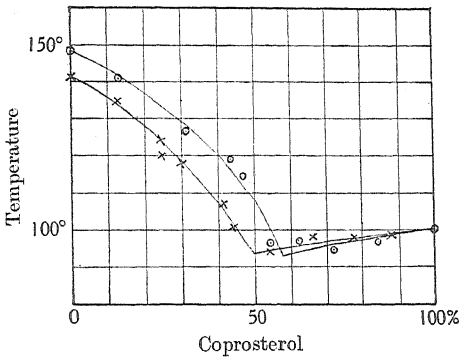

Fig. 1. ×—× Dihydrocholesterol-coprosterol; ⊙—⊙ Cholesterol-coprosterol.

It is a common experience that crude coprosterol preparations, even when extracted with xylene from digitonides precipitated in presence of bromine, show a melting-point of about 80°, thus indicating that the substances in question are not simple mixtures of coprosterol and dihydrocholesterol.

Summary.

The tests for purity of coprosterol have been discussed in connection with melting-point diagrams for coprosterol-dihydrocholesterol and coprosterol-cholesterol.

The author wishes to acknowledge his indebtedness to Prof. R. Schönheimer, formerly Head of the Department of Chemistry in the Pathological Institute, Freiburg, for many suggestions during the progress of this work.

REFERENCES.

Bondzynski and Humnicki (1896–97). *Z. physiol. Chem.* **22**, 396.
Dam (1925). *Biochem. Z.* **158**, 76.
Dorée (1913). *Biochem. J.* **7**, 619.
Fischer (1911). *Z. physiol. Chem.* **73**, 204.
Gardner (1921). *Biochem. J.* **15**, 244.
Lettré (1932). *Liebig's Ann.* **495**, 41.
Rheinboldt and Kircheisen (1926). *J. prakt. Chem.* **113**, 348.
Schönheimer (1929). *Z. physiol. Chem.* **180**, 23.
—— (1930). *Z. physiol. Chem.* **192**, 77.
Willstätter and Mayer (1908). *Ber. deutsch. chem. Ges.* **49**, 2199.
Windaus (1916). *Ber. deutsch. chem. Ges.* **49**, 1724.
—— and Uibrig (1915). *Ber. deutsch. chem. Ges.* **48**, 875.
Uibrig (1915). Dissertation, Freiburg.

CXVIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXXVIII. THE METABOLIC PRODUCTS OF *PENICILLIUM MINIO-LUTEUM* DIERCKX. MINIOLUTEIC ACID.

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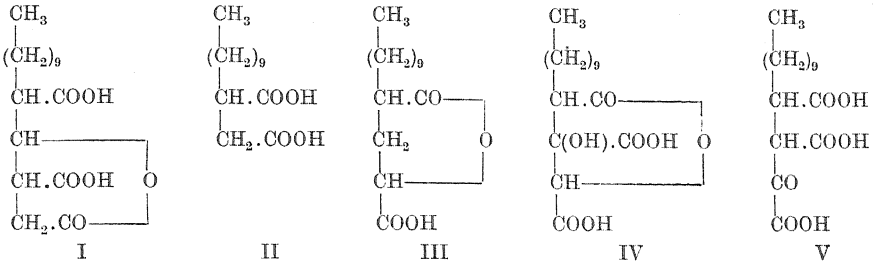
Penicillium minio-luteum Dierckx was described by R. P. Dierckx [1901] and is referred to by Biourge [1923, p. 237] and by Thom [1930, p. 464] in their monographs on the genus *Penicillium*. We have found that when this organism is grown at 24° on Czapek-Dox medium containing glucose as the sole source of carbon a highly pigmented metabolism solution is formed which froths very badly on vacuum-evaporation. The metabolism solution gives heavy amorphous precipitates with aqueous solutions of the salts of many heavy metals, *e.g.* barium, calcium, copper, nickel, lead, iron, and with silver on standing, but gives no precipitate with mercuric salts. Further, on strongly acidifying the metabolism solution a somewhat pigmented crystalline precipitate is formed on standing. Investigation of the barium and copper precipitates and also of the crystalline precipitate formed on acidification has led to the isolation of two colourless crystalline acids: (1) The γ-lactone of γ-hydroxy-$\beta\delta$-dicarboxypentadecanoic acid, $C_{17}H_{28}O_6$ (I). This acid was previously described by Clutterbuck *et al.* [1931] as a metabolic product of *P. spiculisporum* Lehman, and is now for convenience given the name *spiculisporic acid*. (2) A hitherto undescribed, dextrorotatory acid having the empirical formula $C_{16}H_{26}O_7$, for which the name *minioluteic acid* is proposed. It is interesting to note that Thom [1930] places *P. spiculisporum* Lehman and *P. minio-luteum* Dierckx in the *Biverticillata-Symmetrica* group of species of *Penicillium* on morphological grounds.

Minioluteic acid titrates in the cold as a dibasic acid, but a third acidic group is unmasked by heating with an excess of aqueous alkali. Since the third carboxyl group is masked again on acidification, minioluteic acid is obviously a lactonic acid. This view is supported by the fact that minioluteic acid gives a dimethyl ester and a trihydrazide. Since minioluteic acid contains no unsaturated linkages, and apart from the lactone ring must, from its empirical formula, be an open chain compound, it is obviously the monolactone of a dihydroxytricarboxylic acid.

On fusion with potash, oxalic acid, *n*-dodecanoic acid and a dibasic acid having the empirical formula $C_{14}H_{26}O_4$ were obtained. This acid was proved by synthesis to be decylsuccinic acid (II).

On reduction with sodium amalgam, minioluteic acid gives the lactone of a dicarboxylic acid, $C_{15}H_{26}O_4$, which has been shown by synthesis to be the γ-lactone of α-hydroxy-γ-carboxy-*n*-tetradecanoic acid (III).

On the basis of these facts it appears that miniolutеic acid is the γ-lactone of $\alpha\beta$-dihydroxy-$\beta\gamma$-dicarboxy-n-tetradecanoic acid (IV).



Experimental.

Organism.

The organism used in the whole of this work was a culture of *P. minio-luteum* Dierckx (London School of Hygiene and Tropical Medicine Catalogue No. P 44), received originally through the courtesy of Prof. P. Biourge of the University of Louvain in December 1929. It bears Prof. Biourge's Catalogue No. 60, and is actually a subculture of the original organism isolated by Dierckx.

Culture medium and course of metabolism.

The medium used was the ordinary Czapek-Dox solution of the following composition: glucose, 50 g.; $NaNO_3$, 2 g.; KH_2PO_4, 1 g.; KCl, 0·5 g.; $MgSO_4$, $7H_2O$, 0·5 g.; $FeSO_4$, $7H_2O$, 0·01 g.; water to 1000 ml.

Since preliminary experiments showed that better growth and quicker metabolism were obtained at 24° than at 30° the former was used throughout as the temperature of incubation. Batches of 100 one-litre conical flasks, each containing 350 ml. of the above Czapek-Dox solution, were sterilised and sown with a spore suspension of *P. minio-luteum* and incubated at 24°. Growth was fairly rapid at first and produced a whitish mycelium with green areas of conidial growth. As growth proceeded the mycelium became predominantly green with pinkish patches. The metabolism solution, which quickly developed a faint purple colour, gradually became darker in colour until after 5 weeks it was very dark brown. At the end of the incubation period the contents of the flasks in each batch were filtered and treated by one or other of the following methods:

Method A. This method depends on the fact that both miniolutеic and spiculisporic acids are precipitated from aqueous solution by various metallic salts. The filtered metabolism solution and washings, having p_H 4·2–4·3, were treated with a 40 % aqueous solution of barium acetate (128 g.). The precipitate was filtered off and to the filtrate, which now had p_H 5·2, 100 g. of copper acetate dissolved in about 1 litre of hot water were added. The filtrate from the copper precipitate, having p_H 5·0, was treated with a slight excess of aqueous normal lead acetate solution, and the filtrate from this precipitate was brought to p_H 6·2 by the addition of 450 ml. 2N NaOH and precipitated with basic lead acetate solution. The acids contained in the various precipitates were now extracted as follows:

1. *Barium precipitate.* The barium precipitate was ground with an excess of an aqueous solution of sodium sulphate at 60° and filtered hot; the filtrate was acidified with a slight excess of 2N H_2SO_4 and extracted twice with rather less than its own volume of ether. On evaporation of the ether a semicrystalline

solid, weight 46·1 g., remained. On fractional crystallisation from water and decoloration with norite a less soluble fraction, weight 17·3 g., of almost pure minioluteic acid, melting at 170° was obtained. The more soluble fraction was fractionated from a mixture of ether and light petroleum giving 3·7 g. of somewhat impure spiculisporic acid, which on further purification melted at 142–144°. This was identified by comparison with an authentic specimen of spiculisporic acid from *P. spiculisporum*.

2. *Copper precipitate.* The copper precipitate was ground with water, treated with 150 ml. 5*N* HCl in which practically the whole dissolved, and extracted with ether. The ether extract on evaporation gave 37·9 g. of a dark-coloured semi-crystalline mass, from which by fractionation from hot water 17·7 g. of crystalline material were obtained. Nothing but spiculisporic acid (M.P. 144–145·5°) could be isolated from this fraction. No minioluteic acid could be isolated from the copper precipitate.

3. *Lead precipitates.* The normal lead and basic lead precipitates were separately ground with excess of dilute sulphuric acid and filtered from the precipitated lead sulphate, and the filtrate was extracted with ether. The ether extract from the normal lead acetate precipitate proved to be an uncrystallisable syrup from which nothing was isolated. The ether extract from the basic lead acetate precipitate consisted essentially of an uncrystallisable syrup. A small amount (0·6 g.) of a complex mixture of colourless crystalline substances was however isolated, but this has not been further investigated.

Method B. The combined filtered metabolism solution and washings from one batch of 100 flasks were acidified with 3 litres of concentrated HCl and left in the cold room overnight. The precipitated pigmented, crystalline material was filtered off, washed, dried and weighed (80·5 g.). It consisted of a mixture of minioluteic, spiculisporic and possibly other acids, which up to the present have not been isolated in a state of purity. The crude pigmented crystalline mixture was recrystallised six times from hot water (norite), giving 40 g. of colourless material, M.P. 150–156°. Minioluteic acid was separated from this material by taking advantage of the fact that, in contrast with spiculisporic acid, it forms a potassium salt which is insoluble in ethyl alcohol. 40 g. were dissolved in 1 litre of ethyl alcohol, and a solution containing 24 g. of anhydrous potassium acetate in 500 ml. of ethyl alcohol was added slowly and with constant shaking. The precipitated potassium salt was filtered off after half an hour, washed with ethyl alcohol, dissolved in about 900 ml. of warm water and treated with 90 ml. of concentrated HCl. After cooling, the acid was filtered off and dried; yield 34 g., M.P. 164–167°. The alcoholic mother-liquors were evaporated

Table I.

Exp. No.	Incubation period in days	Apparent residual glucose by polarimeter %	Final p_H	Method of isolation	Yields of crude products g.	
1	47	0·23	4·3	A	*Ex* Ba precipitate	46·1
					Ex Cu precipitate	37·9
					Ex basic Pb precipitate	9·0
2	52	0·11	4·2	A	*Ex* Ba precipitate	52·5
					Ex Cu precipitate	37·1
					Ex normal Pb precipitate	4·7
					Ex basic Pb precipitate	13·0
3	50	0·10	—	B		80·5
4	53	0·16	—	B		75·5

in vacuo and dissolved in water, and the acid was precipitated by HCl; yield 3·3 g., M.P. 134–137°. The main fraction melting at 164–167° was now re-precipitated as the potassium salt which was further purified by crystallising once from water. By this means 18·3 g. of pure minioluteic acid melting at 171° were isolated. Pure spiculisporic acid was obtained by fractional crystallisation of the material from the alcohol-soluble potassium salt.

The experimental details of each of four batches of 100 flasks each are given in Table I.

General properties of minioluteic acid.

Minioluteic acid is best purified by crystallisation from hot water from which solvent it separates in colourless needles melting at 171°. (Found by Schoeller: C, 58·00, 57·96; H, 7·65, 7·72 %; OCH_3, nil; N, nil; mol. wt., 317, 332. $C_{16}H_{26}O_7$ requires: C, 58·14; H, 7·93 %. Mol. wt., 330.)

Titration. 0·1576 g. dried to constant weight at 105° and dissolved in 10 ml. ethyl alcohol required 9·55 ml. *N*/10 NaOH corresponding to an equivalent of 165 ($C_{16}H_{26}O_7$ titrating as a dibasic acid requires 165). A further 20·00 ml. *N*/10 NaOH were now added, the mixture heated on the water-bath for 2 hours, cooled and back-titrated with *N*/10 HCl. A further 4·68 ml. *N*/10 NaOH were neutralised during hydrolysis, making a total of 14·23 ml. *N*/10 NaOH and corresponding with an equivalent of 110·7 ($C_{16}H_{26}O_7$ titrating as a tribasic acid requires 110). Minioluteic acid thus titrates in the cold as a dibasic, and on heating as a tribasic acid. 14·23 ml. *N*/10 HCl were now added and the mixture allowed to stand at room temperature for 24 hours. On back-titration 9·48 ml. *N*/10 NaOH were required. These figures show that reversion of the tribasic form to the dibasic form was complete and indicate the probable presence of a γ-lactone ring.

The above conclusions were confirmed by the preparation of the silver salts of the open and closed ring forms which were found to contain 47·80 and 39·35 % Ag respectively. (Theoretical for $C_{16}H_{25}O_8Ag_3$, 48·39 %, and for $C_{16}H_{24}O_7Ag_2$, 39·67 %.)

It was found impossible to isolate the open ring form of minioluteic acid in the free state. A quantity of minioluteic acid was hydrolysed by heating with a measured excess of *N* NaOH. The cooled hydrolysate was shaken with ether and rather less than the theoretical amount of *N*/10 HCl cautiously added with constant shaking, 1–2 ml. at a time. The ethereal solution was treated at room temperature with light petroleum and the material separating was air-dried. It showed an equivalent on titration of 169. (Theoretical for closed form 165, for open form 116.) In a further attempt to prepare the trimethyl ester of the open form of minioluteic acid the ethereal solution as obtained above was methylated directly with diazomethane. The resultant crystalline product proved to be the dimethyl ester, M.P. 86° (see p. 832).

Rotations. (*a*) Free acid in acetone

$$c = 1{\cdot}07.\ [\alpha]^{16}_{5461} + 108{\cdot}1°,\ [\alpha]^{16}_{5790} + 94{\cdot}5°.$$

(*b*) Disodium salt in water, calculated as the free acid.

$$c = 1{\cdot}04.\ [\alpha]^{19}_{5461} + 62{\cdot}4°,\ [\alpha]^{19}_{5790} + 54{\cdot}6°.$$

Solubilities. Minioluteic acid is readily soluble in cold ether, ethyl alcohol, acetone, ethyl acetate, butyl alcohol and hot water. It is somewhat soluble in chloroform (1:400) and is practically insoluble in cold water, benzene and light petroleum.

Salts. Minioluteic acid gives insoluble salts with a large number of metals and in this respect is very similar to spiculisporic acid. A 1 % neutral solution of the sodium salt of minioluteic acid gave heavy precipitates with silver nitrate, ferric chloride, lead acetate and the chlorides of calcium, barium, nickel and zinc; moderate precipitates with copper sulphate and magnesium chloride, and no precipitate with mercuric chloride, uranium acetate and aqueous potassium chloride. In contrast with spiculisporic acid, minioluteic acid gives a potassium salt which is almost insoluble in alcohol, the potassium salt of spiculisporic acid being relatively soluble in this solvent.

Dimethyl ester. Minioluteic acid, dissolved in ether, was treated with an excess of an ethereal solution of diazomethane. Vigorous evolution of nitrogen took place and the ethereal solution was filtered, evaporated and the crystalline residue recrystallised from a mixture of chloroform and light petroleum. Colourless plates, M.P. 86·5°. (Found: C, 60·61, 60·58; H, 8·44, 8·22; OCH_3, 17·41, 17·64 %. $C_{16}H_{24}O_5(OCH_3)_2$ requires C, 60·30; H, 8·44; OCH_3, 17·32 %.)

Trihydrazide. 0·08 g. of the dimethyl ester of minioluteic acid was dissolved in 2–3 ml. ethyl alcohol and 0·16 ml. of 50 % aqueous hydrazine hydrate added. Deposition of micro-crystals began immediately. These were drained, washed with ethyl alcohol and dried *in vacuo* over H_2SO_4. As no suitable crystallising medium could be found the compound was analysed without recrystallisation. The crystals sintered with incipient decomposition at 157–160° and melted finally at 201°. (Found: C, 49·37, 49·22; H, 8·72, 8·60; N, 21·30, 21·30 %. $C_{16}H_{34}O_5N_6$ requires C, 49·19; H, 8·78; N, 21·52 %.)

A saturated aqueous solution of minioluteic acid does not reduce acid potassium permanganate in the cold although the permanganate is slowly reduced on heating. An alcoholic solution of the acid showed no absorption of hydrogen after 45 minutes' shaking with palladised norite. With Wijs's iodine solution 1 g.-mol. of minioluteic acid absorbed 0·845 g.-atom of iodine after 2 hours, but this is almost certainly due to chemical reactions other than the simple addition of iodine. These facts together with the analytical figures obtained for minioluteic acid and its dimethyl ester and trihydrazide indicate the absence from the molecule of the acid of an unsaturated linkage.

Potash fusion of minioluteic acid.

1 g. minioluteic acid was warmed with 5 g. solid KOH and 3 ml. water in a nickel crucible. When dissolution was complete the temperature of the metal-bath was raised slowly to 310° and maintained at this temperature for one hour. The melt was dissolved in water and acidified with dilute H_2SO_4. There was a considerable evolution of CO_2 and a slight smell of butyric acid. The mixture was kept in the cold room overnight and filtered from precipitated material, and the filtrate and precipitate were dealt with separately.

(*a*) *Filtrate.* The filtrate was exhaustively extracted with ether in a continuous extraction apparatus for 30 hours. On evaporation of the ether a crystalline residue, weight 0·41 g., was obtained. This was dissolved in water, titrated with *N* NaOH (equivalent = 61), the neutralised solution was acidified with acetic acid and the oxalic acid removed by precipitation with calcium chloride. Weight of calcium oxalate = 0·50 g. The oxalic acid-free solution was now acidified with H_2SO_4 and re-extracted with ether. The ether solution on evaporation left only a trace of residue from which no succinic or other crystalline acid could be isolated.

(*b*) *Precipitate.* Weight 0·48 g. This was purified by crystallisation from a mixture of ether and chilled light petroleum giving a small quantity (0·06 g.)

of colourless, crystalline plates, M.P. 87–89°, which on recrystallisation from acetone and water melted at 90–91° and had a barely detectable optical rotation. (Found: C, 65·02, 64·85; H, 9·99, 10·12 %. Mol. wt., 246, 250. $C_{14}H_{26}O_4$ requires: C, 65·07; H, 10·16 %; mol. wt., 258.) Equivalent by titration 130. (Theoretical for $C_{14}H_{26}O_4$ titrating as a dibasic acid, 129·1.) This acid was subsequently shown by synthesis to be decylsuccinic acid (II).

It was evident that a second acid was present in the precipitate (*b*). Hence the combined precipitates (*b*) from the potash fusion of 5 g. minioluteic acid were distilled *in vacuo* at 16 mm. with the following results:

Fraction I, collected up to 135°. Weight 0·45 g. Softens at 30°. M.P. 38–40°.

Fraction II, collected from 135 to 145°. Weight 0·57 g. Softens at 34°. M.P. 44–44·5°.

Fraction III. B.P. gradually fell to 100°. Weight 0·39 g. Softens at 41°. M.P. 43–48·5°.

Fraction IV. The residue in the distillation-flask was now distilled at 7 mm. when a fourth fraction distilled at 182°. Weight 0·74 g. M.P. about 58°.

Fraction I was esterified with diazomethane in ether and the residue on evaporation of the ether was treated with 2·7 ml. ethyl alcohol and 1 ml. 50 % aqueous hydrazine hydrate and allowed to stand for 24 hours. A total yield of 0·32 g. of hydrazide was obtained, which was crystallised twice from aqueous alcohol and once from benzene, M.P. 104–105°. A sample of *n*-dodecanoic acid hydrazide prepared similarly melted at 106–107°, and there was no depression in melting-point on mixing the two samples. (Found: C, 67·25, 67·34; H, 11·82, 11·98; N, 13·41, 13·25 %. *n*-Dodecanoic acid hydrazide, $C_{12}H_{26}ON_2$ requires: C, 67·23; H, 12·24; N, 13·07 %.)

Fraction IV, distilling at 182°/7 mm., was recrystallised several times from ether-light petroleum giving finally colourless needles, which after sublimation had M.P. 66–67°. This was shown to be the anhydride of decylsuccinic acid (see below).

Synthesis of decylsuccinic acid (II).

The method adopted was to condense ethyl-α-bromo-*n*-dodecanoate with ethyl sodiomalonate, and then to decarboxylate the tricarboxylic acid obtained on hydrolysis.

*α-Bromo-*n*-dodecyl bromide and ethyl α-bromo-*n*-dodecanoate.* α-Bromo-*n*-dodecyl bromide was synthesised by the method of Auwers and Bernhardi [1891]. *n*-Dodecanoic acid (10 g.) and red phosphorus (0·52 g.) were placed in a flask fitted with a reflux condenser and bromine (14·7 g.) added slowly. The temperature was slowly raised to 90–100°. A little more bromine was added at intervals until the evolution of HBr ceased. The mixture was cooled and the crude product converted, without previous isolation, into ethyl α-bromo-*n*-dodecanoate by the addition of ethyl alcohol (30 ml.). The mixture was kept for some hours, the excess of alcohol removed *in vacuo*, the residue dissolved in ether, washed with water and aqueous sodium carbonate solution, dried over anhydrous Na_2SO_4 and distilled *in vacuo*. The main fraction (13·7 g.) distilled at 115–123°/1 mm.

Triethyl αβ-dicarboxytridecanoate. Sodium (0·88 g.) was dissolved in ethyl alcohol (12 ml.) and ethyl malonate (6·1 g.) added. After half an hour ethyl α-bromo-*n*-dodecanoate (11·7 g.) was added and the mixture heated for 2 hours on the water-bath under reflux. Excess ethyl alcohol was removed by vacuum evaporation, water was added to dissolve the sodium bromide and the mixture was extracted with ether, the extract being dried with Na_2SO_4 and fractionated *in vacuo*. The main fraction (11·5 g.) distilled at 172°/1 mm.

αβ-Dicarboxytridecanoic acid. It was found that the triethyl ester was difficult to hydrolyse completely with alcoholic potash, but satisfactory results were obtained by fusion with potash. The ester was heated with five times its weight of stick potash and three times its weight of water, the temperature of the bath being raised as rapidly as frothing would permit to 300°. The melt was cooled, dissolved in water, acidified, and the beautifully crystalline product filtered off

(M.P. 148°, decomp.). It was recrystallised from a mixture of ether and four volumes of light petroleum. Colourless needles, M.P. 148° (decomp.). (Found: C, 59·27, 59·37; H, 8·56, 8·60 %. $C_{15}H_{26}O_6$ requires: C, 59·57; H, 8·67 %. Equivalent by titration, 100·4. Theoretical for $C_{15}H_{26}O_6$ titrating as a tribasic acid, 100·7.)

Decylsuccinic anhydride. $\alpha\beta$-Dicarboxytridecanoic acid was decarboxylated by distillation *in vacuo*. The product distilled at 160°/2 mm. and solidified in the receiver. It separated from light petroleum in colourless plates, M.P. 65–66°; after sublimation 68·5°. When mixed with the anhydride, M.P. 66–67°, obtained from minioluteic acid (Fraction IV, p. 833) it showed no depression in melting-point. (Found: C, 69·90; H, 9·88 %. $C_{14}H_{24}O_3$ requires: C, 69·93; H, 10·07 %.)

Decylsuccinic acid. Decylsuccinic anhydride (0·50 g.) was heated with 10 ml. *N* NaOH for half an hour on the water-bath. A back-titration with *N* HCl gave an apparent equivalent of 116 (theoretical 120). Sufficient excess of HCl was added to precipitate all the acid. The precipitate (0·5 g.) was recrystallised first from light petroleum and then from aqueous acetone, forming colourless glistening plates, M.P. 90–91°. This melting-point showed no depression on mixing with the dibasic acid, M.P. 90–91°, obtained by potash fusion of minioluteic acid (see p. 833).

Reduction of minioluteic acid with sodium amalgam.

Minioluteic acid (2 g.) was dissolved in 2 *N* NaOH (25 ml.) and water (75 ml.) added. Sodium amalgam (100 g., 2·5 % sodium) was added and the mixture heated on the water-bath. After 4 hours a further 100 g. sodium amalgam were added and so on until a total of 500 g. had been added. From time to time a little concentrated HCl was added to reduce the alkalinity, but the solution was never allowed to become acid. Finally, the mixture was cooled, acidified with HCl, extracted with ether and the ether removed, giving a residue, weight 1·37 g. The residue appeared to be a mixture of substances and was ground and extracted in a Soxhlet with light petroleum, B.P. 60–80°, giving 0·1 g. of a colourless crystalline product, M.P. 114°. The sticky residue which was now difficult to extract was dissolved in absolute alcohol and treated with an alcoholic solution of potassium acetate, giving a precipitate from which no crystalline material could be separated. The alcoholic solution was evaporated *in vacuo* and extracted with cold water. The insoluble portion was dissolved in hot water and acidified. The resultant precipitate, weight 0·16 g., M.P. 113°, was identical with the acid previously extracted with light petroleum, M.P. 114°. The combined fractions were recrystallised from ether-light petroleum giving colourless plates, M.P. 114·5°, which, after sublimation in a high vacuum, was raised to 119°. (Found: C, 66·82, 66·92; H, 9·37, 9·32 %. $C_{15}H_{26}O_4$ requires: C, 66·61; H, 9·70 %.)

0·0856 g. required 3·15 ml. *N*/10 NaOH for neutralisation in the cold, and on heating with excess *N*/10 NaOH required a further 3·37 ml. corresponding to equivalents of 272 and 131. $C_{15}H_{26}O_4$ titrating as a mono- or di-basic acid requires 270 or 135 respectively.

This reduction product, $C_{15}H_{26}O_4$, which is obviously the monolactone of a dibasic acid, thus differs from minioluteic acid, $C_{16}H_{26}O_7$, by one atom of oxygen and one molecule of CO_2. It has been shown by synthesis to be the γ-lactone of α-hydroxy-γ-carboxy-*n*-tetradecanoic acid (III).

*Synthesis of the γ-lactone of α-hydroxy-γ-carboxy-*n*-tetradecanoic acid (III).*

Ethyl decylsuccinate was condensed with ethyl oxalate in the presence of sodium ethoxide in ether, according to the method of Blaise and Gault [1911]. The resulting condensation product was hydrolysed and decarboxylated in alkaline solution and subsequently reduced with sodium amalgam.

Diethyl decylsuccinate. Decylsuccinic acid (16 g.) was esterified in the usual manner with two successive amounts of absolute ethyl alcohol (100 ml.) containing 3 % of HCl. The resultant ester was distilled *in vacuo*, B.P. 155–159°/2 mm., weight 15·9 g.

α-Keto-γ-carboxy-n-*tetradecanoic acid*. Powdered metallic sodium (1·15 g.) was treated with ethyl alcohol (2·3 g.) diluted with 20–30 ml. of ether. The mixture was allowed to stand overnight and ethyl oxalate (7·3 g.) added. After 2 hours ethyl decylsuccinate (15·7 g.) was added and the mixture left at room temperature for 48 hours. The mixture was poured into 20 ml. of 20 % H_2SO_4 and 25 g. of ice, shaken and the ether layer separated. In the method described by Blaise and Gault [1911] for the preparation of α-keto-γ-methylglutaric acid, on which this method is based, the corresponding triethyl ester was extracted from the ether solution at this stage by means of aqueous (25 %) potassium carbonate. A similar procedure in our hands failed to extract any triethyl α-keto-βγ-dicarboxy-*n*-tetradecanoate, probably owing to the feebler acidity of our ester. The ether solution was therefore extracted with *N* NaOH. By this means 6·7 g. of material were extracted whilst 11·7 g. of presumably uncondensed esters remained in the ether. The NaOH solution was now heated at 100° for 14 hours, cooled, acidified and extracted with ether. The ether solution was evaporated, giving a semicrystalline residue (4·2 g.), from which the liquid portion and a quantity of decylsuccinic acid were separated by fractionation from ether-light petroleum. The remainder (1·2 g.) consisted of impure α-keto-γ-carboxy-*n*-tetradecanoic acid. A pure sample of this acid was not prepared, but it was characterised by the preparation of its 2:4-dinitrophenylhydrazone.

2:4-Dinitrophenylhydrazone of α-keto-γ-carboxy-n-*tetradecanoic acid*. This was prepared by Brady's method [1931] from the crude product referred to above and was recrystallised from 70 % aqueous ethyl alcohol. Yellow needles, M.P. 180°. (Found on material dried to constant weight at 100°: C, 54·40; H, 6·62; N, 11·90 %. $C_{21}H_{30}O_8N_4$ requires: C, 54·05; H, 6·49; N, 12·01 %.)

γ-Lactone of α-hydroxy-γ-carboxy-n-*tetradecanoic acid (III)*. The crude acid (0·7 g.) was reduced in alkaline solution with sodium amalgam (400 g., 2·5 % sodium) over a period of 16 hours on a boiling water-bath. The method used was similar to that followed for the hydrolysis and reduction of minioluteic acid. The resultant product was purified by fractionation from ether-light petroleum. Colourless plates. Weight 0·3 g. M.P. 119°, alone or in admixture with the lactone obtained by hydrolysis and reduction of minioluteic acid (p. 834). (Found: C, 66·37; H, 9·66 %. $C_{15}H_{26}O_4$ requires: C, 66·61; H, 9·70 %.)

Summary and discussion.

The metabolic products of *P. minio-luteum* Dierckx, when grown at 24° on Czapek-Dox medium containing glucose as the sole source of carbon, have been investigated. Two aliphatic acids of complex structure have been isolated: (*a*) the γ-lactone of γ-hydroxy-βδ-dicarboxypentadecanoic acid (I) previously described by Clutterbuck *et al.* [1931] as a metabolic product of *P. spiculisporum* Lehman, for which the name spiculisporic acid is now proposed, and (*b*) a hitherto undescribed acid for which the name minioluteic acid is proposed.

The evidence for the molecular constitution of minioluteic acid is briefly as follows:

(1) Minioluteic acid has the empirical formula $C_{16}H_{26}O_7$. It is a saturated dextrorotatory acid and titrates in the cold as a dibasic, and on heating as a tribasic acid. Because of the stability of the lactone ring the latter is probably of the γ-type.

(2) It gives a dimethyl ester and a trihydrazide.

(3) On fusion with potash, oxalic acid, *n*-dodecanoic acid, and decylsuccinic acid (II) $C_{14}H_{26}O_4$ but no succinic acid were isolated. Decylsuccinic acid has been synthesised.

(4) On reduction with sodium amalgam minioluteic acid gives the γ-lactone of α-hydroxy-γ-carboxy-*n*-tetradecanoic acid $C_{15}H_{26}O_4$ (III), the constitution of which has been settled by synthesis. This change involves the loss of a molecule of CO_2 and an atom of oxygen. The above facts are easily interpreted by assigning to minioluteic acid the molecular constitution (IV), *i.e.* the γ-lactone of αβ-dihydroxy-βγ-dicarboxy-*n*-tetradecanoic acid.

The origin of the products given on potash fusion and on reduction with sodium amalgam may be readily explained by postulating the formation of an intermediate product α-keto-βγ-dicarboxy-*n*-tetradecanoic acid (V) (or its corresponding enol form) from miniolueic acid in alkaline solution by loss of a molecule of water. The conversion in aqueous solution of αβ-dihydroxy-compounds, particularly of aliphatic nature, into the corresponding aldehyde or ketone by loss of a molecule of water is a well-known reaction [Lieben, 1902].

If the intermediate formation of α-keto-βγ-dicarboxy-*n*-tetradecanoic acid (V) be accepted the origin of the products isolated becomes obvious. Since the keto-group in this compound is in the β-position with regard to the β-carboxy-group this substance should behave as a β-keto-acid. By analogy with aceto-acetic acid, which on hydrolysis with strong potash gives rise to two molecules of acetic acid, α-keto-βγ-dicarboxy-*n*-tetradecanoic acid (V) under similar conditions (*i.e.* potash fusion) should yield oxalic and decylsuccinic acids. The latter may be presumed to break down further in presence of oxygen and afford *n*-dodecanoic and oxalic acids in the same way as succinic acid, which under these conditions gives oxalic acid. This accounts for all the degradation products isolated after subjecting miniolueic acid to potash fusion.

On hydrolysis with dilute alkali α-keto-βγ-dicarboxy-*n*-tetradecanoic acid (V) would be expected to give rise to α-keto-γ-carboxy-*n*-tetradecanoic acid and CO_2, by elimination of the β-carboxy-group, just as acetoacetic acid under these conditions affords acetone and CO_2. Reduction with sodium amalgam would then convert the keto-group into a hydroxy-group, the final product being α-hydroxy-γ-carboxy-*n*-tetradecanoic acid (III). This hypothesis is confirmed by the method used for synthesis of α-hydroxy-γ-carboxy-*n*-tetradecanoic acid since the decarboxylation and subsequent reduction of α-keto-βγ-dicarboxy-*n*-tetradecanoic acid, which must here be the first product of synthesis, follows the same course. The two typical methods of breakdown of a β-keto-acid are thus represented.

A comparison of the constitutional formulae assigned to spiculisporic and miniolueic acids reveals the fact that both are mono-γ-lactones of tricarboxylic acids. Considering for simplicity the hydrolysed, non-lactonic forms, it is seen that both contain the *n*-dodecanoic acid grouping, but whereas in spiculisporic acid this is linked by the α-carbon through a carbinol group with succinic acid, in miniolueic acid it is linked, again by the α-carbon, to dihydroxysuccinic (*i.e.* tartaric) acid, but without the intervention of a carbinol group.

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REFERENCES.

Auwers and Bernhardi (1891). *Ber. deutsch. chem. Ges.* **24**, 2224.
Biourge (1923). *La Cellule*, **33**, 237.
Blaise and Gault (1911). *Bull. Soc. Chim.* (4), **9**, 458.
Brady (1931). *J. Chem. Soc.* 756.
Clutterbuck, Raistrick and Rintoul (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**, 301.
Dierckx (1901). *Ann. Soc. Sci. Bruxelles*, **25**, 83.
Lieben (1902). *Monatsh. Chem.* **23**, 60.
Thom (1930). The Penicillia, 464. (London: Baillière, Tindall and Cox.)

CXIX. NOTE ON THE LOSS OF NITROGEN AND SULPHUR ON DRYING FAECES.

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When the collection of faeces during a metabolic experiment extends over a considerable period of time, difficulties arise in the determination of the total nitrogen and sulphur contents. Sampling of the wet composite specimen is generally unsatisfactory and ordinary methods of drying on a steam-bath result in a considerable loss of both nitrogen (as ammonia) and sulphur (mainly as sulphide).

If nitrogen is the only element under consideration its loss as ammonia can be prevented by acidification with H_2SO_4 before drying. To obviate drying Van Slyke [1932] has recommended sampling from a suspension of the faeces in H_2SO_4. If sulphur is also being determined, the excess of sulphuric acid which requires to be added masks the sulphur originally present in the faeces, and further, acidification leads to loss of H_2S and certain organic sulphides on drying.

The following experiments indicate the extent of the nitrogen and sulphur loss during drying. A method of preventing this loss is described.

Experimental.

Total N was estimated by the Kjeldahl method; total S by Denis's modification of Benedict's ashing method. Analyses were performed in triplicate, first on the wet specimens and then on aliquot portions subjected to various procedures. The accuracy of the sampling of these wet specimens was confirmed by the close agreement of triplicate estimations.

All specimens of human faeces were analysed within an hour or two of being voided.

Exp. 1. Sample of faeces containing 0·8976 g. total N had after 10 days' continuous drying on a steam-bath 0·8439 g. N. % loss = 5·9.

Exp. 2. Sample of faeces containing 0·616 g. N had after 14 days' drying on a steam-bath 0·558 g. N. % loss = 9·4.

Exp. 3. Sample of faeces containing 1·599 g. N and 0·222 g. S contained after 14 days' continuous drying on a steam-bath 1·531 g. N and 0·116 g. S. % loss N = 4·2. % loss S = 47.

Exp. 4. Fresh wet faeces containing 1·305 g. N were transferred to a vacuum-pan and the temperature of the bath raised to 80°. Ammonia-free air was passed over the faeces and drying was carried out under reduced pressure. In the course of 1 hour during which the temperature of the bath was rising no ammonia came over. In the next 5 hours 0·0286 g. N was trapped in $N/10$ H_2SO_4. That the loss of N was as NH_3 was confirmed by nesslerisation. During the subsequent 6 hours only 0·0007 g. more N came over. Loss of N = 2·2 % in course of 14 hours' drying.

In an attempt to prevent the simultaneous loss of N and S from faeces by means of a preliminary treatment with substances which would trap NH_3 and H_2S no simple method could be devised. It was eventually determined that the most satisfactory method was to moisten the faeces with excess 2 % copper acetate and trap the ammonia in acid during the process of drying.

Exp. 5. Sample of faeces containing 0·114 g. S contained after drying with copper acetate 0·115 g. S. There was thus no loss of S. When an aliquot portion was dried without any precaution for a similar period, *viz.* 2 days, the dried faeces contained 0·109 g.—a loss of 4·8 % S.

From clinical and bacteriological considerations it is obvious that the amounts of free gases contained in faeces must vary considerably.

Exp. 6. A sample of faeces containing 1·867 g. N was dried in the presence of 2 % copper acetate for 24 hours. Ammonia-free air was passed over the faeces and then through standard H_2SO_4. Ammonia was estimated by nesslerisation as it was found that acetic acid was also driven over. The total amount of trapped N was 0·275 g. The total residual N was 1·591 g. 1·866 g. N of the original 1·867 g. N were thus accounted for.

The original sample of faeces contained 0·1705 g. S. An aliquot dried without precaution for 24 hours contained 0·147 g. S and one dried for 6 days contained 0·1355 g. S—losses of 14 % and 20 % respectively. An aliquot treated with copper acetate lost no S on drying for 24 hours.

Exp. 7. A sample of faeces containing 2·522 g. N was dried in presence of 2 % copper acetate for 48 hours. Ammonia-free air was passed over the faeces and through a 10 % NaOH trap to absorb the acetic and other volatile acids. The air was finally passed through standard acid. In the final estimation the residual ammonia was distilled over from the NaOH solution and added to that trapped in the acid. The total loss was 0·0339 g. N. The nitrogen content of the dried faeces was 2·495 g. The original 2·522 g. were thus accounted for.

The original sample of faeces contained 0·588 g. S. An aliquot dried without precaution for 24 hours contained 0·581 g. S and one dried for 5 days 0·5675 g. S—losses of 1·2 % and 3·5 % respectively. An aliquot dried with 2 % copper acetate lost no S on drying for 24 hours.

In these experiments allowance was made for the trace of S in the copper acetate by adding aliquot amounts at the appropriate times. It was thus added to ordinary air-dried specimens of faeces after the process of drying was complete but before analysis.

It is seen from these experiments that an accurate analysis of the nitrogen and sulphur of faeces is possible if in the process of drying, ammonia-free air is passed over the faeces to which have been added a dilute solution of copper acetate in excess, the air is then passed through alkali to trap acetic and other volatile acids and then through standard acid. The alkaline fluid traps some ammonia and this is finally distilled into the excess standard acid.

SUMMARY.

1. The process of drying faeces on a water-bath leads to a considerable loss of N (mainly as ammonia) and S (mainly as sulphide).

2. This loss can be prevented by adding an excess of a dilute solution of copper acetate to the faeces before drying them in a closed vessel through which passes a current of dry ammonia-free air. The ammonia liberated from the faeces during drying is trapped in acid.

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REFERENCE.

Van Slyke (1932). Quantitative clinical chemistry, **2**, 77. (Baillière, Tindall and Cox, London.)

CXX. THE FATTY ACIDS OF PIG LIVER.
I. THE OCTADECENOIC ACIDS AND THE DESATURATION THEORY.

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The accumulation of fat which occurs in the liver under the action of many substances, which is seen in numerous clinical conditions and which may be produced also by dietary methods, is the outstanding proof of the fact that the liver plays a fundamental part in fat metabolism. The desaturation theory, associated with the names of Leathes and Hartley, according to which fatty acids of the depot fat are transported to the liver, where they are desaturated and passed on to the tissues in this more readily oxidisable form, was put forward in an attempt to interpret this phenomenon. This theory is based on two lines of evidence: the effect of the food fat on the nature of the liver fatty acids [Leathes and Meyer-Wedell, 1909] and the investigations of Hartley [1909] on the unsaturated acids of the liver and depot fat of the pig. Considerable evidence has been put forward recently which suggests that the basis on which the desaturation theory rests is far less secure than had appeared. The demonstration by Leathes and Meyer-Wedell [1909] that the iodine value of the liver acids of cats and rats, which had been fed on highly unsaturated fish oils, might exceed that of the acids of the oil administered, was interpreted as evidence for desaturation. The extensive work of Sinclair [1932, 1, 2] seems to lend considerable support to the suggestion made by Joannovics and Pick [1910] that the increase in iodine value of the liver fatty acids caused by feeding oils of high iodine value observed by Leathes and Meyer-Wedell was due not to desaturation but to preferential absorption of the more unsaturated acids from the portal circulation. Sinclair has shown that the iodine value of the fatty acids of the liver phosphatides is rapidly raised by the administration of very small amounts of a highly unsaturated fat such as cod-liver oil, whilst the lowering of this high value by substitution of the cod-liver oil by a relatively saturated fat takes a much longer time. These and many other results of Sinclair imply an ability on the part of the tissues and particularly of the liver preferentially to take up the more unsaturated acids from the fat present in the food and tenaciously to retain them. At the present time therefore the selective retention by the liver cells of the more unsaturated acids seems probable and, if this can be established, one of the lines of evidence on which the desaturation theory rests will be invalidated. The second and perhaps more conclusive work which points to liver desaturation of fatty acids was provided by Hartley [1909] who isolated from the liver acids of the pig a $\Delta^{12,13}$ oleic acid and a $\Delta^{9,10;12,13}$ linoleic acid, while the ordinary $\Delta^{9,10}$ oleic acid was absent. Neither of these two former acids was present in lard which, however, was found to contain

$\Delta^{9,10}$ oleic acid and stearic acid. Hartley concluded that the liver cells possessed the power of introducing a double bond into the 12 : 13 position, so that on mobilisation to the liver stearic acid was converted into $\Delta^{12,13}$ oleic acid and similarly $\Delta^{9,10}$ oleic acid was converted into $\Delta^{9,10;12,13}$ linoleic acid.

This attractive evidence seems strong support for the liver desaturation theory, especially as the $\Delta^{12,13}$ oleic acid was unlikely to have arisen from the food fat, for such an acid has not been found in natural products. It was perhaps surprising, therefore, when Turner [1930] failed to obtain any evidence for the presence of this particular oleic acid in sheep liver, in which only $\Delta^{9,10}$ oleic acid was found to be present. In spite of the conflicting evidence which the work of Sinclair seems to provide and the doubt suggested by the results of Burr and Burr [1929; 1930; Burr *et al.*, 1932], which will be mentioned later, confirmation of the proof of the presence of this $\Delta^{12,13}$ oleic acid in liver would yet provide strong evidence for the desaturation hypothesis. We have undertaken an investigation of the fatty acids of pig liver with this object in view.

Experimental.

Preparation of the liver fatty acids.

The preparation of the crude fatty acids was carried out industrially in the following manner. 100 kg. of pig liver obtained direct from the slaughter-house were minced and mixed with a solution of potassium hydroxide. The mixture was allowed to stand until dissolution had occurred, when it was extracted six times with a non-chlorinated industrial solvent mixture. The material left on evaporation of the solvent (unsaponifiable matter and unhydrolysed lipoid substances) was saponified with alcoholic potash and the unsaponifiable fraction removed. The alkaline liver liquors (320 l.) were acidified with hydrochloric acid and the fatty acids removed by solvent extraction. The soap solutions resulting from the saponification and extraction of the unsaponifiable fraction were acidified and treated similarly. The crude fatty acids as received by us weighed 4·37 kg. and contained a considerable amount of the higher-boiling fractions of the solvent used for extraction. Contaminating impurities were removed by dissolution of the crude material in light petroleum (40 l.) and filtration after standing overnight. Evaporation of the light petroleum was followed by removal of the bulk of the industrial solvent by distillation on a boiling water-bath at 1 mm. The partially purified material was then saponified in the usual way with alcoholic potassium hydroxide. Removal of the unsaponifiable fraction by extraction with ether removed also the remainder of the industrial solvent. The fatty acids after purification by treatment with light petroleum weighed 1·835 kg. (I.V. 110, mol. wt. 229). The yield, 1·84 % of the fresh liver weight, is probably about 60 % of the total acids originally present in the liver in different forms. The extraction of acidified liver tissue after hydrolysis is an extremely difficult operation, especially in bulk, and the yield obtained therefore is to be regarded as satisfactory. Such a yield makes it unlikely that the material is not representative of the whole.

Removal of the saturated acids.

The oxidations described later were carried out after the removal of the saturated acids either from the bulk preparation described above or from various small laboratory preparations made by exhaustive alcohol extraction of minced liver with subsequent preparation of the fatty acids from the alcoholic extract. The Twitchell process, as modified by Hilditch and Priestman [1931], applied

to 5·033 g. of the bulk preparation (I.V. 110, mol. wt. 299) gave 1·675 g. of saturated acids (I.V. 2·5, yield 33·3 %). A similar result was obtained on the large scale. 670 g. of the same acids yielded 231 g. of saturated acids (I.V. 2, mol. wt. 277, yield 34 %) and 440 g. of unsaturated acids (I.V. 153, mol. wt. 321). The proportion of saturated acids in the other preparations was of the same order, the highest figure being 39 %.

The preparation of the dihydroxy-acids from the unsaturated fraction.

Since the main object of this work was to establish the structure of the acids containing one double bond, it was desirable to explore the available methods for oxidation in order to determine the conditions under which greatest yields of dihydroxy-acids could be obtained. By oxidation of pure oleic acid in 0·1 % solution with alkaline potassium permanganate at 0° for 5 minutes, Lapworth and Mottram [1925, 1] obtained 95 % of the theoretical yield of dihydroxystearic acid. These authors state that the yield is depressed if the oxidation is carried out in the presence of more than a certain maximum of less saturated acids. The liver unsaturated acids consist of a complex mixture of acids from oleic acid to acids of more than twenty carbon atoms and having four and five double bonds. When such a mixture is oxidised under the conditions in which the greatest yield is obtained from pure oleic acid, the yield of dihydroxy-derivatives may clearly be seriously diminished. In order to determine the optimum conditions a series of comparative experiments, in all of which oxidation by alkaline permanganate has been used, have been carried out on the unsaturated fraction itself, and also on the material remaining after various processes have been carried out in attempts to concentrate the acids containing one double bond. Before describing these experiments, mention is made of the fact that when a mixture of acids, which has been freed from unsaponifiable matter, is oxidised by alkaline permanganate, there are produced substances which may be extracted by ether from the alkaline solutions of the oxidation products. The nature of this unsaponifiable fraction generated during oxidation has not been investigated. It is a complex mixture derived from the more unsaturated acids. It has been removed at various stages of the oxidations and it is mentioned here to explain the appearance of unsaponifiable fractions in the results which follow.

The method of Lapworth and Mottram [*1925, 1*]. 19·07 g. of the unsaturated fraction (I.V. 193) from a small laboratory preparation of fatty acids were dissolved in 20 l. of ice-cold water containing 14·0 g. potassium hydroxide. 14·6 g. potassium permanganate dissolved in 700 ml. ice-cold water were now added quickly with stirring, and after 5 minutes the oxidation was stopped by the addition of concentrated hydrochloric acid and sodium bisulphite solution. After removal by filtration, the insoluble oxidation products were dried *in vacuo* at room temperature over phosphorus pentoxide and extracted with light petroleum (B.P. 80–100°) to remove unoxidised acids and unsaponifiable material. The undissolved residue consisting of di- and tetra-hydroxy derivatives was then separated into its two main components by boiling with ether, the tetra-derivative being practically insoluble in that solvent. The portion soluble in light petroleum (6·54 g.) had I.V. 59·3 and mol. wt. 444. From it 1·03 g. of unsaponifiable matter were removed, yielding a mixture of acids of mol. wt. 383 and I.V. 69. 4·38 g. of these acids soluble in light petroleum were now re-oxidised under exactly the same conditions. The oxidation products insoluble in water

were filtered off, dried and extracted with petroleum. The petroleum-soluble portion was oxidised a third time. The combined yields of the various products from the three oxidations were: dihydroxy-derivatives 2·06 g.; tetrahydroxy-derivatives 0·70 g.; a semi-solid mixture of saturated acids soluble in petroleum 2·393 g. Allowing for the removal of portions of the oxidation products for analytical purposes, the yield of dihydroxy-derivatives was 13·0 % of the unsaturated acids or 8·0 % of the original mixed acids from the liver.

The method of Hartley [*1909*] *used by Turner* [*1930*]. The essential difference in the method used by these workers, as compared with that of Lapworth and Mottram, was that they carried out their oxidations in solutions which were approximately 13 times more concentrated. Following their procedure, 9·28 g. of the unsaturated acids (I.V. 193) were dissolved in 92 ml. 4 % potassium hydroxide, 280 ml. water were added and, after cooling to 0°, the mixture was oxidised by the addition of 400 ml. of 1·5 % potassium permanganate solution also at 0°. The oxidation was terminated after 10 minutes by treatment as in the previous experiment. A second oxidation of the petroleum-soluble fraction of the oxidation products was carried out under similar conditions. The final yields were: dihydroxy-derivatives 0·20 g.; tetrahydroxy-derivatives 0·15 g. This amount of dihydroxystearic acid corresponds to 2·5 % and 1·5 % of the unsaturated and of the original mixed acids respectively. The corresponding figures for the Lapworth and Mottram process were 13·0 and 8·0 %. The latter process, carried out at great dilution, is clearly far more efficient for the quantitative isolation of hydroxy-derivatives of the singly unsaturated acids present in liver. Lapworth and Mottram's statement as to the effect of the presence of more highly unsaturated acids in depressing the yield of dihydroxy-acid from oleic acid in a mixture made it likely that this yield of 13 % might be considerably below the value obtainable if the more highly unsaturated acids were first removed. For this reason attempts were made to increase the yield by removing as much of the unsaturated acids as possible from the mixture. For this purpose, two methods were employed.

Removal of more unsaturated acids. When certain of the more unsaturated acids are brominated, isomeric soluble and insoluble bromides are produced. The latter may be removed and the soluble product debrominated. On rebromination a further amount of insoluble bromide is formed. This principle was employed in an attempt to free the acids containing one double bond from the more unsaturated acids, alternate bromination and debromination being employed until no further insoluble bromide could be obtained. 19·56 g. of the unsaturated acids (I.V. 193) brominated in ether at 0° yielded 7·48 g. of ether-insoluble bromo-acids and 35·58 g. of soluble products. The latter were dissolved in light petroleum (B.P. 40–60°) and further insoluble bromides, which were precipitated after standing for 24 hours at 0°, were removed. The petroleum-soluble fraction, 31·57 g., contained 47·1 % Br. 30·34 g. of this product on debromination by the method of Rollet [1909] yielded 14·05 g. of fatty acid of I.V. 132. On rebromination of 13·85 g. of these acids, a further 1·21 g. of ether-insoluble bromide was removed. The remainder (Br 43 %), which remained completely soluble in light petroleum after standing for 2 days at 0°, was debrominated as before. By repetition of this bromination-debromination process, there were finally obtained 10·62 g. of fatty acids of I.V. 98. 10·1 g. of the latter acids when oxidised by the method of Lapworth and Mottram yielded 1·98 g. of dihydroxy-products (M.P. 130° indefinite) and 0·429 g. of tetrahydroxy-products (M.P. 168° indefinite) the former yield corresponding to 10·9 % of the original unsaturated acids, as compared with a yield of 13 % by direct oxidation

of the unsaturated acids without preliminary application of the bromination process. Unsuccessful attempts were also made to apply the Twitchell process to the mixture of soluble brominated acids in an endeavour further to remove the polybromo-acids.

The second method used for removing the more unsaturated acids was the lithium soap process which has been frequently described in the literature in various forms (in particular Moore [1919]). 22·15 g. of unsaturated acids from the large preparation (I.V. 153) were dissolved in 90 ml. absolute alcohol and 1·8 g. lithium hydroxide dissolved in 22·5 ml. water were added. After standing at 0° overnight, about 3 g. of lithium soaps were removed. A further 1 g. of lithium hydroxide dissolved in 100 ml. 80 % alcohol was added and the mixture maintained at 0° for 24 hours. The combined alcohol-insoluble lithium soaps yielded 6·01 g. of acids (I.V. 96·1, mol. wt. 286, 27 % of the total) while 15·6 g. of the more unsaturated acids (I.V. 171, mol. wt. 327, 70·4 % of the total) were obtained by decomposition of the soluble soaps. 4·755 g. of the more saturated acids yielded on oxidation by the method of Lapworth and Mottram 2·68 g. of dihydroxy-compounds (2·40 g., M.P. 127°; 0·28 g., M.P. 112°) together with 0·21 g. tetrahydroxy-derivatives (M.P. 168°). 10·72 g. of the more unsaturated fraction yielded by similar oxidation 1·23 g. dihydroxy-compound (M.P. 126°) and 0·05 g. of tetrahydroxy-derivatives. For comparison with these results a sample of the original unsaturated acids (I.V. 153) was directly oxidised without being subjected to the lithium treatment. The results of this experiment are summarised and compared with others in Table II.

The effect of increasing the permanganate concentration on the yield of the oxidation products. In the oxidation experiments so far described, the proportions of the reagents used by Lapworth and Mottram were rigidly adhered to. Since the starting material used by these workers was oleic acid (I.V. 90·4), whereas the acids used in the present work were far more unsaturated, it was considered desirable to investigate the effect on the yield of the dihydroxy-fraction of increasing the permanganate concentration proportionally to the increase in the iodine value.

The results of all these variations of the conditions of oxidation on the yields of the products are set out in Tables I and II. Table I shows that the yield of dihydroxy-acids by the Lapworth and Mottram method is about five times as great as that obtained by Hartley's method, while the yield of tetrahydroxy-compounds is about two and a half times as great. The separation of the more unsaturated acids by bromination has given poorer yields of both products when compared with the direct oxidation by the former method. Increase in the permanganate concentration causes a fourfold increase in the yield of the tetrahydroxy-fraction (Exp. 6), but slightly depresses the yield of dihydroxy-products. Table II shows a slightly increased yield of the two products as a result of the lithium soap treatment (*cf.* 4 and 5). The increase in the amount of the dihydroxy-fraction, which was the main objective, was however insufficient to compensate for the extra labour involved in the lithium soap separation, and accordingly direct oxidation of the unsaturated acids was employed in the large scale preparation. Tables I and II show the variations which may occur in the proportions of singly unsaturated acids present in the mixed acids from different liver samples. In Exps. 2 and 5, in which the oxidations were carried out under identical conditions, but on different samples of acids, the yields of dihydroxy-products were 8·0 and 14·8 % respectively of the original mixed acids. Hartley [1909] obtained yields of dihydroxy-compounds varying from 2·6 to 4·5 % of the mixed pig liver acids.

Table I. *Yields of di- and tetra-hydroxy-acids obtained under various conditions of oxidation.*

The unsaturated acids used in these experiments formed 61·5 % of the total mixed acids, and had I.V. 193.

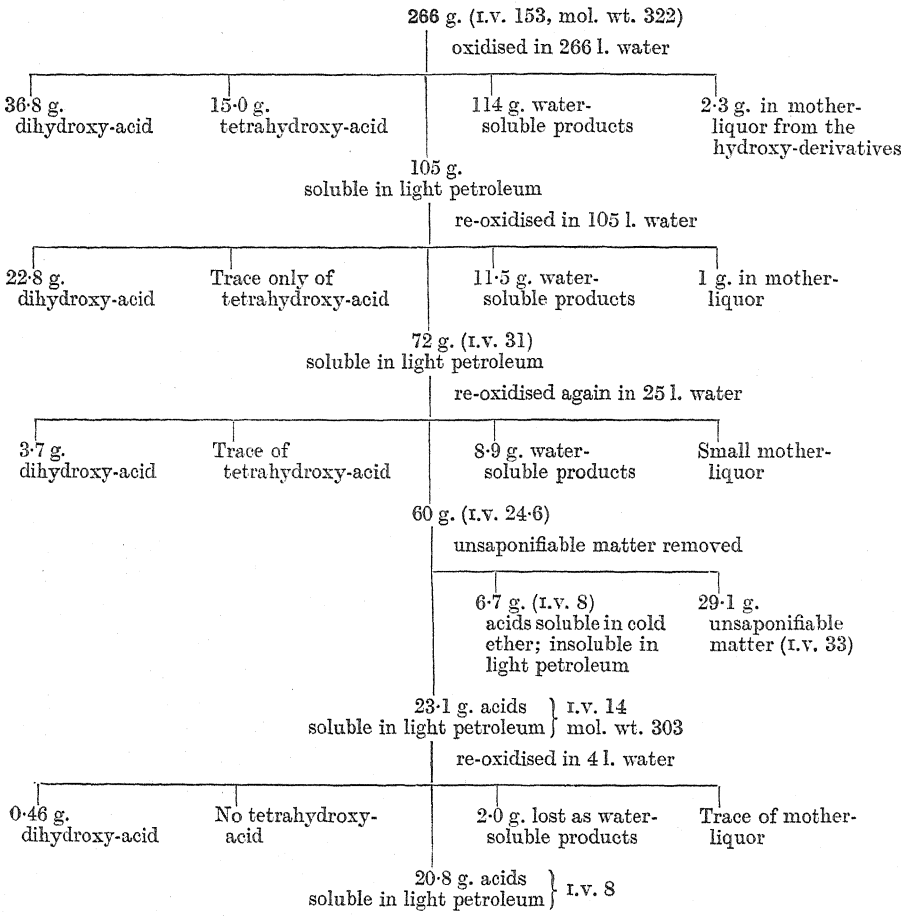
Method used	% yield from unsaturated acids		% yield from original acids (calc.)	
	Di-hydroxy	Tetra-hydroxy	Di-hydroxy	Tetra-hydroxy
1. Hartley's method of oxidation of unsaturated acids	2·5	1·6	1·5	1·0
2. Lapworth and Mottram's method on the unsaturated acids	13·0	4·2	8·0	2·6
3. The singly unsaturated acid in the mixed unsaturated acids first concentrated by bromination experiments and then oxidised by Lapworth and Mottram's process	11·0	2·4	7·0	1·5

Table II. *Yields of di- and tetra-hydroxy-acids obtained by the Lapworth and Mottram method.*

The unsaturated acids used in these experiments constituted 66·1 % of the original liver acids and had I.V. 153.

Particular acids oxidised	% yield from acids from lithium soaps		% yield from total unsaturated acids		% yield from original mixed acids (calc.)	
	Di-hydroxy	Tetra-hydroxy	Di-hydroxy	Tetra-hydroxy	Di-hydroxy	Tetra-hydroxy
4. Acids from insoluble lithium soaps	56·3	4·4	15·65	1·2	10·34	0·8
Acids from soluble lithium soaps	11·5	0·5	8·3	0·33	5·5	0·2
Total by this method	—	—	23·95	1·53	15·84	1·0
5. Direct oxidation of mixed unsaturated acids	—	—	22·4	1·0	14·8	0·7
6. The same as the preceding, except that the permanganate has been increased	—	—	21·0	4·1	13·9	2·7

The preparation of the dihydroxy-acids on a large scale. 266 g. unsaturated acids (I.V. 153, mol. wt. 322) were oxidised in 266 l. water at 4°, with the proportions of potassium hydroxide and permanganate recommended by Lapworth and Mottram. The second oxidations of the material soluble in light petroleum were carried out in exactly similar manner. In the third and fourth oxidations the volume of water was decreased because of the low iodine value of the petroleum-soluble fractions which indicated that not more than a part of them could be oleic acid. The successive stages in the procedure are best illustrated by the scheme (p. 846), which is followed by a brief summary of the final results.



N.B. Water-soluble products are calculated by difference.

The results are summarised in Table III.

Table III.

Wt. of unsaturated acids used	266 g.,	equivalent to	402 g. of original mixed acids
,, crude dihydroxy-derivatives	63·8 g.,	,,	56·9 g. of "oleic" acid
,, crude tetrahydroxy-derivatives	15·0 g.,	,,	12·1 g. of "linoleic" acid
,, saturated acids	20·8 g.		
,, acids soluble in cold ether, insoluble in light petroleum*	6·7 g.		
,, unsaponifiable matter*	29·1 g.		
,, water-soluble products *etc.* (by difference)†	140·4 g.		

* The nature of these oxidation products was not investigated further.

† These products would consist mainly of a mixture of the further cleavage products of small molecular weight derived from all the different unsaturated acids, together with the hydroxy-derivatives of the highly unsaturated acids (C_{20} and C_{22}).

Investigation of the dihydroxy-fraction.

In view of the possibility that the three yields of dihydroxy-acids obtained in the successive oxidations, 36·8, 22·8 and 3·7 g. respectively, might differ in nature on account of preferential oxidation of one or more acids, each was

separately fractionally crystallised from a mixture of alcohol:ether (1:1). The yields and properties of the resultant six fractions are recorded in Table IV.

Table IV. *Fractionation of the dihydroxy-acids.*

	Fraction	Weight g.	M.P. °C.	C %	H %	Mol. wt. (titration)
From first oxidation 36·8 g.	1*a*	28·7	126 –126·5	67·75	11·3	314
	1*b*	5·2	119·5–122 (indef.)	67·1	11·2	314
From first re-oxidation 22·8 g.	2*a*	15·3	129·5–130·5	68·4	11·45	312
	2*b*	5·6	117·5–120·5	68·5	11·4	315
From second re-oxidation 3·7 g.	3*a*	2·1	128·5–129	68·5	11·5	317
	3*b*	1·2	117·5–120·5	68·6	11·4	315
$C_{18}H_{36}O_4$ requires				68·35	11·5	316

The analytical figures for fractions 1*a* and 1*b* suggest the presence of some tetrahydroxy-acid caused by the slight solubility in the very large volumes of boiling ether which were used in the separation of the crude di- and tetrahydroxy-acids from each other. These fractions were accordingly combined, extracted with the minimum volume of boiling ether and the soluble dihydroxy-product was then fractionally crystallised from alcohol-ether. Following the removal in this way of 5 g. of crude tetrahydroxy-acid from fraction 1, all the fractions were successively crystallised from this solvent, and by this means the material was finally divided into a series of fractions with the following compositions and properties:

Table V. *Composition and properties of the dihydroxy-acids.*

Fraction No.	Weight g.	M.P. °C.	C %	H %
1	36·0	130·5–131	68·4	11·3
2	1·3	128	68·1	11·3
3	0·8	124–125	68·4	11·6
4	8·1	117–120	68·45	11·4
5	1·7	114	67·7	11·2
$C_{18}H_{36}O_4$ requires			68·35	11·5

Table V shows that at least 70 % of the dihydroxy-acids consist of a compound melting at 130·5–131°. The melting-points and combustion analyses of fractions 2–5 imply the existence of three compounds: one, M.P. 130·5–131° (fraction 1); another of low carbon content, M.P. 114° (fraction 5); and an intermediate compound which constitutes the main bulk of the material, apart from fraction 1, namely fraction 4, 8·1 g., M.P. 117–120°. Fraction 4 was repeatedly recrystallised from various solvents, but no effective further purification was obtained. This fraction appears to be a mixture of two dihydroxystearic acids, while fraction 5 is possibly the dihydroxy-derivative of an acid of lower carbon content.

The constitution of dihydroxystearic acid, M.P. 130·5–131°.

This was investigated by the method of Lapworth and Mottram. To 10 g. of the acid dissolved in 6 l. water containing 30 g. potassium hydroxide cooled to below 6° were added 4 l. 1 % aqueous potassium permanganate also cooled to below 6°. The mixture was well shaken and after remaining at room temperature for 4 hours the oxidation was stopped by the addition of sodium bisulphite solution and concentrated hydrochloric acid. A slight precipitate,

formed at this stage was filtered off. It weighed 0·12 g. and melted at 126° and was therefore probably unchanged dihydroxystearic acid. The amount of acid actually oxidised was therefore 9·88 g. The aqueous filtrate after neutralisation with sodium carbonate was reduced to a volume of 900 ml. and was then extracted thoroughly with ether.

Monobasic acid. The residue left after the removal of the ether was distilled in steam for 3 hours. The distillate was extracted with light petroleum (B.P. 40–60°). The solution, first dried over sodium sulphate, yielded on evaporation of the solvent 3·965 g. of liquid acids. 3·81 g. of these acids were distilled. 3·332 g. distilled at 234°. Further fractionation yielded 2·334 g. having B.P. 238° and mol. wt. (determined by the silver salt method) 144·2, 142·6. The zinc salt was prepared (found: C, 54·7; H, 8·5 %). The small amount of acid boiling above 240° was not further investigated. The theoretical figures for *n*-octanoic and *n*-nonanoic acids are as follows:

	Yield g.	B.P. ° C.	Mol. wt.	Of zinc salt C %	Of zinc salt H %
n-Octanoic acid	4·50	236–237	144	54·6	8·6
n-Nonanoic acid	4·91	253–254	158	56·9	9·0

Dibasic acid. The residue from the distillation in steam (about 70 ml.) was filtered while hot from traces of tar and the dibasic acid allowed to crystallise; yield 4·29 g., M.P. 135–136° with previous softening. Suberic acid requires 5·44 g., M.P. 141°. On concentrating the aqueous filtrate, a further 0·28 g., M.P. 120° (indefinite) was obtained. These products were divided into four fractions by crystallisation from ether.

1. 2·522 g. M.P. 140·5–141°; mol. wt. 174·0; C, 55·3; H, 8·1 %.
2. 1·083 g. M.P. 140–141°.
3. 0·218 g. M.P. 135–137°.
4. 0·27 g. M.P. 100° indefinite.

Suberic acid, $C_8H_{14}O_4$, requires C, 55·15; H, 8·1 %; mol. wt. 174; M.P. 141°. The fourth fraction was converted to its magnesium salt, which was crystallised from water. The least soluble portion yielded 0·05 g. of acid of M.P. 102·5°. Azelaic acid melts at 106°. The oxidation products were therefore suberic and *n*-octanoic acids, together with small amounts of the acids having one more carbon atom, *i.e.* azelaic and *n*-nonanoic acids. In their oxidation of pure oleic acid, Lapworth and Mottram [1925, 2] obtained an 80 % yield of suberic acid and a 70 % yield of *n*-octanoic acid. They did not isolate azelaic or *n*-nonanoic acids from their oxidation products. They also estimated the amount of oxalic acid produced from the carbon atoms adjacent to the double bond. This was not sought in our experiments. These results show therefore that at least 70 % of the singly unsaturated acid present in this sample of pig liver is $\Delta^{9,10}$ octadecenoic acid.

Investigation of the dihydroxystearic acid, M.P. 117–120°.

4·048 g. of this product were oxidised and the products isolated in exactly the same way as in the previous experiment. None of the substance remained unoxidised.

The monobasic acid. This weighed 1·505 g.; mol. wt. 145. 1·252 g. on distillation gave three fractions, (*a*) 0·5 g., B.P. up to 220°; (*b*) 0·41 g., B.P. 221–235°; (*c*) 0·27 g., B.P. 236–240°. By redistillation of fractions (*a*) and (*b*) there were

obtained (i) 0·31 g., B.P. 220°; (ii) 0·28 g., B.P. 221–235°. The mol. wt. of fraction (i), determined by the silver salt method, was 136·4. This together with its boiling-point precludes the presence of *n*-hexanoic acid. The zinc salt was prepared from fraction (*b*) and gave on analysis C, 55·5; H, 8·8 %. The theoretical yields and the mol. wts. and boiling-points of the possible acids which might be present are:

Acid	Yield	Mol. wt.	B.P. ° C.
n-Nonanoic	2·02	158	253–254
n-Octanoic	1·84	144	236–237
n-Heptanoic	1·66	130	224
n-Hexanoic	1·49	116	206
Crude product (found)	1·505	145	up to 240

With the small amount of material available, it was not possible to carry the investigation further. Study of the boiling-points and molecular weights of the various fractions, together with the initial weight of the crude oxidation product, make it appear certain that *n*-hexanoic acid could not have been present unless in traces. The mixture of acids probably consisted of *n*-heptanoic, *n*-octanoic and *n*-nonanoic acids.

The dibasic acid. The aqueous residue from the distillation in steam was filtered from 0·24 g. of a dark brown oil and from the filtrate there crystallised 1·73 g. dibasic acid, M.P. 126–129°, clearing finally at 132°. On concentrating the mother-liquor, a second crop of 0·28 g. separated, M.P. 120° (indefinite). Total crude dibasic acid, 2·0 g. The theoretical yields of sebacic, azelaic and suberic acids would have been 2·59 g., 2·41 g. and 2·23 g. respectively. It is to be noted that in both crops the product differed in melting-point from that obtained at the corresponding stage from the oxidation of the higher-melting dihydroxystearic acid (p. 848). On recrystallisation from ether, the first crop still melted at 120–125° and had mol. wt. 175. Further recrystallisation from ether had no apparent effect. Both fractions were therefore converted into their magnesium salts and these fractionally crystallised from water. The acids from the most and from the least soluble salts were obtained and recrystallised both from water and from ether. The most soluble magnesium salt gave a dibasic acid, which, after recrystallisation, weighed 0·32 g.; M.P. 139–141°; C, 55·15; H, 8·1 %; mol. wt. 175 (by titration). The acid from the least soluble magnesium salt weighed 0·28 g., crystalline plates, M.P. 133–134°; C, 59·60; H, 8·9 %; mol. wt. 206. The theoretical data for four possible dibasic acids are:

	M.P. ° C.	C %	H %	Mol. wt.
$C_{11}H_{20}O_4$	124	61·1	9·3	216
$C_{10}H_{18}O_4$	134	59·4	8·9	202
$C_9H_{16}O_4$	106	57·4	8·5	188
$C_8H_{14}O_4$	141	55·15	8·1	174

The dibasic products of oxidation therefore contain suberic and sebacic acids; and it follows that in all probability azelaic acid is a third component, because, apart from any other consideration, suberic and sebacic acids present alone in a mixture would be very readily separated, whereas these two components were only obtained in the present instance with difficulty. The presence of other dibasic acids in very small traces is also possible. In Table VI are given the products which would arise from a number of acids oxidised under the conditions used by us. The presence of suberic and *n*-octanoic acids and also of part of the azelaic and *n*-nonanoic acids in the oxidation products in this experiment shows

Table VI.

Acid	Main products	By-products
9:10-Dihydroxystearic acid	Suberic and *n*-octanoic acids	Azelaic and *n*-nonanoic acids
10:11 „	Azelaic and *n*-heptanoic acids	Sebacic and *n*-octanoic acids
11:12 „	Sebacic and *n*-hexanoic acids	Nonamethylenedicarboxylic acid and *n*-heptanoic acid
12:13 „	Nonamethylenedicarboxylic acid and *n*-pentanoic acid	Decamethylenedicarboxylic acid and *n*-hexanoic acid

that the lower-melting dihydroxy fraction is a mixture and that the $\Delta^{9,10}$ dihydroxy-derivative has not been entirely removed by fractional crystallisation. The yields and analytical data suggest that about one-half of the fraction may be the 9 : 10 compound. It therefore remains to account for the apparent formation of sebacic and *n*-heptanoic acids. Under the conditions of oxidation used, the presence of these products suggests that the hydroxy-groups were originally in the 10 : 11 positions. This view, however, can only be put forward with considerable caution. Until the oxidation products can be obtained in far greater quantity to enable their isolation in pure form, no definite statement can be made. These results do however indicate the presence of an acid which is neither $\Delta^{9,10}$ nor $\Delta^{12,13}$ octadecenoic acid, and which probably forms not more than 10 % of the total dihydroxy-fraction.

Oxidation of ordinary oleic acid. At this stage it was felt wise to prepare the dihydroxystearic acid from ordinary oleic acid, in order to see whether it could be fractionated into high- and low-melting products of a similar nature. Accordingly 14·27 g. oleic acid (which still contained a little saturated acid) were oxidised by Lapworth and Mottram's method and yielded 13·27 g. dihydroxystearic acid, M.P. 128°. Allowing for the 1·67 g. of saturated acids which were removed by light petroleum, the yield was 94·0 % of theory. On fractionating the hydroxy-derivative as in the large experiment on p. 847 there were obtained 10·35 g., M.P. 130°; 1·0 g., M.P. 128° and 0·47 g., M.P. 122°, the mother-liquor of 1·4 g. being a semi-liquid product. This fractionation was very similar in its results to that described for the hydroxy-compounds from liver, except that in this case there was no corresponding lower fraction, with a melting-point of 117–120°, which remained unchanged after several recrystallisations.

Discussion.

These results show that at least 80 % of the octadecenoic acid of pig liver is ordinary oleic acid ($\Delta^{9,10}$), while some 10 % may be the $\Delta^{10,11}$ acid. No evidence was obtained of the presence of the $\Delta^{12,13}$ acid obtained by Hartley [1909]. This latter result is the more striking because the $\Delta^{12,13}$ acid was the only acid found in pig liver by that author. It was isolated by him as the dihydroxy-acid, M.P. 129°, which was further oxidised. From the steam-volatile oxidation products, calcium and barium salts were prepared and these contained the percentages of metal required by theory for *n*-hexanoic acid. It is to be noted however that the decamethylenedicarboxylic acid, which should have been produced under the conditions of oxidation used by him along with *n*-hexanoic acid, was not isolated. Hartley contrasts the melting-point of his dihydroxy-acid, M.P. 129·5°, with that obtained by Edmed [1898] by oxidation of oleic acid, which gave a dihydroxy-acid, M.P. 134°, as evidence for the dissimilarity of his compound from the $\Delta^{9,10}$ acid. Robinson and Robinson [1925], however, who record M.P. 132° for 9:10-dihydroxystearic acid prepared from

pure oleic acid, comment that the melting-points (up to 136°) previously recorded in the literature are too high.

Little can be said at present concerning the small fraction of the acid which was isolated in this work as a dihydroxy-acid, M.P. 117–120°. The material available in the work carried out by Hartley [1909] and Turner [1930] did not allow them fractionally to crystallise their dihydroxy-products, and no corresponding compound was obtained.

We feel that our results further weaken the available evidence for desaturation of fatty acids by the liver, for it will be recalled that Turner [1930] failed also to obtain evidence of the presence of any octadecenoic acid in sheep liver other than the $\Delta^{9,10}$ acid. Bearing in mind the extensive researches of Sinclair [1932, 1, 2] which show the remarkable avidity with which the liver removes unsaturated acids from the portal circulation and the tenacity with which it retains them when the unsaturated fat in the diet is substituted by a saturated one, the original results of Leathes and Meyer-Wedell can be interpreted equally well for the present as being due to preferential absorption of fatty acids or as being due to desaturation.

From another direction also considerable evidence is accumulating which seems to cast doubt on the validity of the desaturation hypothesis. The extensive literature which is accumulating to show that animals will not grow on a diet which is rigorously freed from fat, but that normal growth is restored when very small amounts of linoleic or linolenic acids are administered, has given rise to the conception of "essential" fatty acids which implies a very limited ability of the animal to synthesise certain unsaturated acids at least [Burr and Burr, 1929; 1930; Burr *et al.*, 1932]. If this finding is substantiated, it is of particular importance to emphasise that one of the "curative" acids is linoleic acid, which on the desaturation hypothesis should be produced from depot oleic acid by introduction of a double bond into the 12:13 position.

Summary.

1. With a view to obtaining further evidence on the theory of desaturation of fatty acids in liver, the octadecenoic acids of pig liver have been investigated by oxidation of the unsaturated acids and subsequent further oxidative degradation of the dihydroxy-acids.

2. Description is given of experiments carried out with a view to increasing the yields of dihydroxy-acids when the mixed unsaturated acids are oxidised with alkaline permanganate.

3. Fractional crystallisation of the latter acids shows the presence of at least two octadecenoic acids. One of these is shown to be $\Delta^{9,10}$ octadecenoic acid (ordinary oleic acid) and to constitute at least 85 % of the octadecenoic acids present. The second dihydroxy-acid, M.P. 117–120°, yielded on further oxidation a mixture of monobasic and dibasic acids which could not be satisfactorily separated on account of the small amount of material available. The evidence so far obtained from study of the oxidation products suggested the presence of a $\Delta^{10,11}$ acid.

4. The $\Delta^{12,13}$ octadecenoic acid isolated by Hartley [1909] from pig liver was shown to be absent.

5. The bearing of these results on the liver desaturation theory is discussed.

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REFERENCES.

Burr and Burr (1929). *J. Biol. Chem.* **82**, 345.
—— —— (1930). *J. Biol. Chem.* **86**, 587.
—— —— and Miller (1932). *J. Biol. Chem.* **97**, 1.
Edmed (1898). *J. Chem. Soc.* **73**, 627.
Hartley (1909). *J. Physiol.* **38**, 353.
Hilditch and Priestman (1931). *Analyst*, **56**, 364.
Joannovics and Pick (1910). *Wiener klin. Woch.* **23**, 573.
Lapworth and Mottram (1925, 1). *J. Chem. Soc.* **127**, 1628.
—— —— (1925, 2). *J. Chem. Soc.* **127**, 1987.
Leathes and Meyer-Wedell (1909). *J. Physiol.* **38**, *Proc.* xxxviii.
Moore (1919). *J. Soc. Chem. Ind.* **38**, 320.
Robinson and Robinson (1925). *J. Chem. Soc.* **127**, 175.
Rollet (1909). *Z. physiol. Chem.* **62**, 410.
Sinclair (1932, 1). *J. Biol. Chem.* **95**, 393; **96**, 103.
—— (1932, 2). *J. Biol. Chem.* **100**, lxxxvii.
Turner (1930). *Biochem. J.* **24**, 1327.

CXXI. THE PHOSPHATIDES OF THE WHEAT GERM.

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Investigations of the phosphatides of cabbage leaves have shown that the main constituent is phosphatidic acid, present as its calcium salt, and that lecithin and kephalin are absent [Chibnall and Channon, 1927]. This finding suggested that salts of phosphatidic acid might be the intermediate compounds in the synthesis of lecithin and kephalin from triglycerides, according to the scheme postulated by Trier [1912], and suggested further studies designed to throw light on this question. The work described in this paper was the first part of an investigation planned in order to study the phosphatides of a seed and to trace the changes which occur on germination. While it was in progress investigations along similar lines were reported by Smith and Chibnall [1932] and Jordan and Chibnall [1933]. The former workers identified lecithin, kephalin and the calcium or magnesium salt of phosphatidic acid as constituents of the phosphatide mixture obtained from grass; while the researches of the latter, designed along lines similar to our own, were concerned with an exhaustive study of the distribution of phosphatidic acid, lecithin and kephalin in the runner bean during the different stages of germination and growth. They established for the first time the presence of phosphatidic acid in a seed. Because of the difficulty of obtaining adequate amounts of material for fractionation, we chose wheat germ as our starting material, for it can be obtained commercially and by far the greater part of the phosphatide of the wheat grain is present in the embryo.

Experimental.

Extraction of the wheat germ.

As a result of studies of the phosphatide content of seeds, Schulze and Steiger [1889] recommended extraction by alcohol at 60°, the extract being evaporated to dryness and the residue extracted with ether, because they found that extraction of seeds directly with ether did not remove all the phosphatide. The advantage of the increased phosphatide yield obtained by this method tends to be offset by the fact that the final ethereal extract is contaminated by non-phosphatide substances, some of which may contain phosphorus. Jordan and Chibnall [1933] submitted the cotyledon residues of runner beans which had been already extracted with ether to this process. They found that much of the additional ethereal extract so obtained consisted of substances which readily lost their phosphorus on shaking an ethereal solution with dilute acid, and they consider that the extra amount of true phosphatide extracted by alcohol is not more than one-quarter of the total. With these findings in mind, we carried out an extensive series of extractions with ether, light petroleum, alcohol and acetone, alone and in mixtures, in order to obtain an extract of high phosphatide content and uncontaminated as far as possible. The degree

to which the phosphorus obtained in the extracts existed as non-phosphatide phosphorus was systematically investigated by the methods which were used for the large preparation and are described later, and only the method finally adopted as a result of these experiments need be described. Alcohol-ether (1:1) was found to be the most useful solvent for extraction, and a series of comparative experiments on one sample of wheat germ in which different volumes were employed was carried out. The germ was mixed with the solvent and the mixture heated to boiling. After standing for several hours, at room temperature, the mixture was filtered and the residue washed with successive volumes of ether until the filtrate was colourless (usually 4 volumes). The filtrate was evaporated to dryness *in vacuo* on a water-bath at 60° and the residue dissolved in anhydrous ether. After several hours' standing, the white sediment was removed by filtration and the ether-soluble material obtained by evaporation. The figures recorded below are typical of the results obtained:

Solvent	Volumes	Yield % of germ	P as % of germ
Ether (Soxhlet)	—	7·40	0·019
Alcohol-ether (1:1)	6	7·60	0·028
	9	7·80	0·025
	12	8·25	0·035
	20	8·53	0·036
	(mean from 6 different samples)		

Since our object was to obtain the phosphatide fraction in as great a yield as possible, extraction with 12 volumes of alcohol-ether as described above was finally adopted throughout this work. The yield of oil by this method was 8·40 % (mean of seven samples). Its phosphorus content corresponded to its containing about 10 % of phosphatide.

Precipitation of the phosphatide.

The removal of the phosphatide quantitatively from an oil in which it is present in relatively low concentration is difficult without the addition of inorganic salts. Because of a desire to avoid use of the latter, investigations were made of the optimum conditions for precipitation from ethereal solution by acetone. Samples from one preparation of wheat germ oil were dissolved in varying volumes of ether and to these solutions were added 9 volumes of acetone calculated on the volume of oil used. After cooling in the ice-box for several hours, the precipitates were filtered off and washed with acetone. From a phosphorus determination on the precipitate, the recoveries under the varying conditions were calculated. These are recorded in Table I. As a result of this experiment we adopted the method of dissolving the oil in 1·5 volumes of ether followed by precipitation with 9 volumes of acetone.

Table I. *Percentage of recovery of phosphatide on precipitation of ethereal solutions by acetone.*

	Ether-soluble P as % of the wheat germ	Phosphatide (P × 25) as % of oil or precipitate	Recovery of ether-soluble P in precipitate %
Original extract	0·0232	7·8	—
Precipitation by acetone (9 vols.) after dissolution in ether:			
Vols. 1	0·0202	54	87
1·5	0·0208	60	90
2	0·0191	60	82
3	0·0171	65	73

Analysis of a series of samples.

Before proceeding with the bulk preparation, a series of extracts from different samples of germ (400–850 g. each) was prepared by these methods in order to study the variations likely to be encountered. Their analyses are recorded in Table II.

Table II. *Analysis of seven samples of wheat germ oil.*

Sample	A	B	C	D	E	F	G	Mean
Age (days)	100 (kept at 4°)	42 (kept at 0°)	1	1	2	1	1	—
Yield g./100 g. germ	9·80	8·90	7·7	7·4	8·5	9·1	7·8	8·37
Acid value	—	—	—	17·5	21·7	14·1	16·1	17·6
Saponification value	181	188	190	192	184	180	—	184
I.V.	110	111	115	113	111	107	—	111
P % of extract	0·49	0·55	0·34	0·29	0·32	0·43	0·35	0·39
Fatty acids % of extract	83·2	80·0	82·3	84·7	83·4	82·6	83·8	82·8
I.V.	121	122	112	119	122	124	128	120
Mol. wt.	284	281	282	284	278	278	284	284
Unsaponifiable matter:								
Yield % of extract	6·8	8·0	9·2	5·6	6·7	6·1	6·8	7·03
Sterol (by digitonin)	55	50	47	68	60	65	—	57·5
I.V.	99	109	109	103	108	106	—	105·8

From Table II calculations may be made as to the approximate composition of the extract. Later in this paper it is shown that the phosphatide is present as calcium, magnesium and potassium phosphatidates, together with lecithin and kephalin, and that little of the phosphorus of the extract is non-phosphatide-phosphorus. If the mixed phosphatides be regarded as having a mean phosphorus content of 4 %, the approximate composition of the extract calculated on the mean values in Table II is:

Phosphatide $\left(\frac{0{\cdot}39}{4{\cdot}0} \times 100\right)$	9·75 %
Free fatty acid $\left(\frac{17{\cdot}6}{5{\cdot}6} \times 28{\cdot}2 \times 100\right)$	8·8 %
Glyceride (total fatty acids − phosphatide acids $(18{\cdot}2 \times \mathrm{P})$ − free fatty acid) × 1·045	69·9 %
Unsaponifiable matter	7·03 %
Total	95·48 %

Thus about 4·5 % of the extract appears to be contaminating material of non-fatty nature. Analysis of the phosphatide fraction gave the results recorded in Table III.

Table III. *Analysis of the acetone-insoluble fractions from seven samples of wheat germ oil.*

	A	B	C	D	E	F	G	Mean
Yield as % of extract	13·7	15·9	8·0	10·2	10·4	18·7	12·6	12·8
Yield as % of germ	1·3	1·4	0·6	0·8	0·9	1·7	1·0	1·1
I.V.	77	78	74	78	78	75	—	77
Fatty acids %	55·3	52·6	57·2	57·7	53·3	56·4	—	56·2
I.V.	122	121	123	118	125	120	132	123·0
N %	1·8	1·8	1·5	1·5	1·5	1·6	1·5	1·60
P %	2·9	2·9	2·8	2·4	2·6	2·6	2·3	2·65
Ash %	2·4	2·6	—	2·7	3·2	2·6	—	2·70

The figures in Table III show that the mean value of the N:P ratio is 1·34, indicating the presence of contaminating nitrogenous substances which are so often found in the phosphatide-containing ethereal solutions of the residue left after evaporation of an alcoholic extract of tissues. Calculation on the basis of 4 % phosphorus shows that the mean value for the maximum amount of phosphatide possibly present is 66·25 %. This would yield on hydrolysis 48·32 % of fatty acid, calculated as oleic acid (P × 18·2), compared with 56·2 % found. The calculations from Table II, which indicate the presence of 4·5 % of non-lipoid substances in the original extracts, thus seem borne out by the presence of about 30 % of non-phosphatide in the acetone-insoluble fractions. Since these latter constituted 12·7 % of the original extracts, the 4·5 % would become 36 % if all the extraneous material of the extract were precipitated with the phosphatides. The phosphorus recovery in the acetone precipitates corresponds to 86·3 % of that originally present in the extracts. It is to be observed that the I.V. of the acids obtained by hydrolysis is identical with that of those yielded by the original material, which suggests that the composition of the phosphatide acids does not differ markedly from that of the glyceride acids.

Large scale preparation. 9·5 kg. of wheat germ, received fresh and kept in cold storage for 5 days, were extracted with 114 l. of ether-alcohol (1:1) in six batches. The preparation, carried out as described in 8 days, yielded 770 g. (8·5 %) of a brown oil which gave the following analytical values: acid value, 18·0; fatty acids (I.V. 125, mol. wt. 282), 85·3 %; unsaponifiable matter, 5·4 %; P, 0·39 %; all of which figures correspond closely with those recorded as the mean value in Table III.

Precipitation of the phosphatide.

Acetone (6·5 l.) was added to a solution of 747 g. in ether (1·2 l.). After standing for 15 hours in the refrigerator, the crude phosphatide was filtered off and washed with acetone. It was redissolved in ether (135 ml.) and reprecipitated by the addition of acetone (420 ml.). The product after washing with acetone weighed 68·9 g. (9·2 % of the extract). It analysed as follows: P, 2·97 %; N, 1·52 %; amino-N, obtained by Van Slyke apparatus after hydrolysis for 9 hours with *N* sulphuric acid, removal of the fatty acids and neutralisation after concentration, 0·15 %; the ash, 4·27 %, contained 29 % P and 12 % Mg. In order to ensure the absence of unsaponifiable matter which might have been carried down during the phosphatide precipitation, 0·517 g. was hydrolysed with sodium ethoxide for 4 hours. The yield of unsaponifiable matter was 0·032 g. or 6·20 %. This corresponds to 10·5 % of the unsaponifiable fraction present in the original extract. The fatty acids obtained after removal of the unsaponifiable fraction weighed 0·290 g. (56·1 %). These acids had I.V. 120 and mol. wt. 299.

From these data, and assuming that the ash represents pyrophosphate derived from metallic salts of phosphatidic acid present in the original mixture, an approximate distribution of the phosphorus may be derived as follows:

P present originally as salts of phosphatidic acid $\left(\text{ash } 4{\cdot}27\ \% \times \frac{29}{100}\right)$ ...	1·24 %
P present as kephalin $\left(\text{amino-N } 0{\cdot}15\ \% \times \frac{31}{14}\right)$	0·33 %
Residual P (2·97 − 1·24 − 0·33)	1·40 %
The residual P if present as lecithin would require $1{\cdot}4 \times \frac{14}{31}$ % N	0·63 %
The non-phosphatide-N, 1·52 − 0·78	0·74 %

On the basis of these figures, the composition of the mixture would be: lecithin, 36·4 %; kephalin, 8 %; phosphatidate calculated as the magnesium salt, 28·9 %; unsaponifiable matter, 6·2 %; giving a total of 79·5 %. Hence the fraction contains 20·5 % of extraneous substances which have a mean content of 3·6 % N. A phosphatide mixture such as the above would yield on hydrolysis 54·24 % fatty acids as against 56·1 % found. The phosphorus present in the phosphatide precipitate corresponds to not more than 70 % of that present in the original extract as compared with the mean figure of 86·3 % for samples A–G. We have no explanation to advance for this considerably lower figure.

Evidence was next sought as to the validity of the assumption concerning the nature of the ash and thereby whether the excess nitrogen as shown by the N:P ratio of 1·14:1 was due to extraneous nitrogenous substances present in the fraction or to the presence of a diaminophosphatide. The process finally adopted was that of emulsification of the phosphatide in water followed by aggregation by the addition of acetone and salt, a modification of that used by MacLean [1911]. 1·9 g. of the phosphatide, which will be referred to henceforth as P1, were emulsified in 200 ml. water, and 200 ml. acetone were added. The precipitate P2, 0·5579 g., was removed by filtration; to the filtrate sodium chloride was added until further precipitation ceased. This precipitate P3 weighed 0·976 g. The analyses of P2 and P3 were so similar that they can be grouped together and compared with those of P1.

Table IV. *Recovery after precipitation of the phosphatide emulsion by acetone.*

	P2 and P3	P1	% recovery referred to P1
Yield % of P1	80·7	—	—
Ash	5·23	4·27	98·86
N	0·86	1·52	45·66
P	3·29	2·97	89·19
Fatty acids	63·2	57·0	91·18

Several deductions can be made from Table IV. In the first place the emulsification process has removed 54·34 % of the original nitrogen with a relatively small loss (10·81 %) of the phosphorus and has yielded a product having an N:P ratio of 1:2·61. Further, this mild treatment, which would largely remove water-soluble inorganic material, including inorganic phosphate, and which would not cause any appreciable hydrolysis of the phosphatide, has not removed the original inorganic constituents, for there is a virtually complete recovery of the ash on incineration. The N:P ratio and the nitrogen content (N 0·86 %) definitely show that the phosphatide mixture contains non-nitrogenous phosphatides, for not more than 50 % of lecithin (N 1·74 %) can be present. There is thus strong presumptive evidence for the presence of phosphatidic acid. 0·86 % N, calculated as lecithin, would require P 1·93 % and fatty acids 35·1 %, leaving 1·36 % P and 28·1 % of fatty acids to be accounted for. 1·36 % P would represent 33 % of the fraction if present as the alkaline earth salts of phosphatidic acid and would yield 25 % of fatty acids on hydrolysis. With the 6·2 % of unsaponifiable matter present in P1, 89 % of the fraction can be thus tentatively assigned. Thus this process has left in the product some 10 % of impurity. (Calculation also shows that the 19·3 % of material, removed as water-soluble products, has N 3·78 %, P 1·66 %, fatty acids 26·0 %. Such an amount of phosphorus would correspond to 43 % of it being present as lecithin or similar phosphatide, which would require 0·75 % N and would yield 30·2 % of fatty acids on hydrolysis.)

This experiment suggests therefore that the phosphatide mixture consists of lecithin and kephalin, together with about two-thirds of the sum of their amounts as salts of phosphatidic acid, and the method adopted provides an excellent means for concentrating the phosphatide with relatively little loss.

Before treating the main bulk of material, further preliminary work was carried out with a view to determining the best conditions for the isolation of phosphatidic acid. The original method used by Channon and Chibnall [1927] and also used by Chibnall and his co-workers was to shake the mixed phosphatides in ethereal solution with dilute hydrochloric acid. After washing the solution with water, the residue left after evaporation of the solvent was separated into a lecithin-kephalin fraction and phosphatidic acid by virtue of the solubility of the latter in acetone. The phosphatidic acid was then further purified by conversion into the lead salt. We encountered two difficulties when we attempted to carry out this process with our material. The first of these was the production of persistent emulsions when the mixed phosphatides were shaken in ethereal solution with aqueous acid, due to the lecithin and kephalin present. These were not encountered in the work on cabbage where the latter phosphatides were absent, but are commented on by Smith and Chibnall [1932] in their grass studies. This difficulty which rendered quantitative separations impossible, was overcome by a method which should prove of general use in phosphatide studies. We have used in place of water a mixture of water 100, acetone 40, sodium chloride 5. Acid may be added to the mixture as required and for final washing the salt may be omitted if desired. This has been found an extremely useful device, for separations are usually both rapid and quantitative.

The second difficulty was that we were unable to separate phosphatidic acid from the mixed acid-washed phosphatides by precipitation of the lecithin-kephalin fraction with acetone. Many variations in this procedure were attempted, but studies of the nitrogen and phosphorus percentages in a large number of fractions (an example is fraction P4 given later) showed that little separation was obtainable by this method. We have no suggestion to offer in explanation, save that perhaps the nature of the fatty acids present in the phosphatidic acid, or the position of attachment of the phosphoric acid to the glycerol molecule may be involved. The method adopted to overcome this difficulty has been to shake the phosphatide mixture, after washing with acid, directly with lead acetate. The lecithin fraction was then largely separated from the lead salts by repeated extraction with absolute alcohol. This process was only effective because the percentage of kephalin in our product was low.

In the fractionation which follows, the theoretical analytical figures below will serve for comparison with the results described. Any phosphatide preparation is a mixture of compounds varying in the nature of the fatty acids, and these calculated figures given are based on compounds containing two molecules of oleic acid.

	N	P	C	H	Mol. wt.	Fatty acids yielded by hydrolysis %	Ash as pyrophosphate %
Lecithin	1·74	3·86	65·75	10·71	803	70·3	—
Kephalin	1·88	4·17	66·08	10·48	743	75·9	—
Calcium phosphatidate	—	4·20	63·33	9·88	739	76·3	17·18
Lead phosphatidate	—	3·42	51·66	8·06	906	62·3	32·45
Magnesium phosphatidate	—	4·29	64·71	10·09	723	78·1	15·36
Potassium phosphatidate	—	4·00	60·31	9·15	775	72·7	21·28
Phosphatidic acid	—	4·42	66·76	10·41	701	80·5	—

Fractionation of the main preparation.

1. *Treatment by emulsification in water and precipitation by acetone.* 30·8 g. P1 (N, 1·52; P, 2·97 %) were emulsified in 500 ml. water and, after adding acetone (500 ml.) and 12·5 g. sodium chloride, the mixture was chilled for 15 hours. The precipitate P2, 27·1 g. (N, 0·94 %; P, 3·28 %), was treated in similar manner with half the quantities of water, salt and acetone. The precipitated phosphatide P3 weighed 25·38 g. (N, 0·87; P, 3·26 %). The first treatment thus removed 45·36 % of the nitrogen; the small amount removed by the second treatment, 7·28 %, was taken as evidence that further repetition of the procedure would not materially improve the product. The total loss of phosphorus into the aqueous phase was 11·5 %.

2. *Treatment with acid.* 25·1 g. P3 dissolved in 300 ml. ether were shaken successively with 300, 150 and 150 ml. of a mixture of water 400 ml., acetone 200 ml., sodium chloride 40 g., with sufficient hydrochloric acid added to make the solution $N/20$. The ethereal solution was washed five times with the acetone-brine solution without added acid, 700 ml. in all, and yielded on evaporation 24·4 g.

3. *Separation by ether-acetone.* 24·3 g. dissolved in ether (80 ml.) were precipitated with acetone (480 ml.). The process was repeated three times. The combined mother-liquors were evaporated to dryness and, by chilling the product after dissolution in hot acetone, a further precipitate was obtained. The total acetone-insoluble fraction P4 was 22·65 g.; the soluble fraction P5 weighed 1·64 g.

The yields and the analyses of these four products and that of P1 were:

	Yield g.	C %	H %	N %	P %
P1	30·8	60·88	9·56	1·52	2·97
P2	27·1	62·93	9·69	0·94	3·28
P3	25·38	60·42	9·31	0·87	3·26
P4	22·65	58·82	9·39	0·93	3·54
P5	1·64	71·25	11·32	0·25	1·53

4. *Treatment with lead acetate.* (*a*) *Acetone-soluble fraction P5.* 1·56 g. dissolved in 70 ml. ether were shaken with 20 ml. aqueous lead acetate solution containing a little acetic acid. The ethereal solution after four washings with water yielded 1·66 g. This was dissolved in 10 ml. ether and the lead salt precipitated by adding 30 ml. alcohol. The lead salt was again dissolved in ether and reprecipitated by alcohol. The twice precipitated lead salt P6 weighed 0·57 g. The soluble residue 1·09 g. was discarded.

(*b*) *Acetone-insoluble fraction P4.* In an attempt to avoid the emulsions encountered under (*a*), a saturated lead acetate solution in 50 % aqueous acetone was employed. 17·8 g. P4 dissolved in 180 ml. ether were shaken with 180 ml. of this lead acetate solution and the mixture was centrifuged. The ethereal solution was washed in the centrifuge-cups four times with 40 % aqueous acetone. On evaporation this yielded 19·5 g. while from the emulsion 2·43 g. were obtained; total yield 21·93 g. The product was extracted three times with hot absolute alcohol, 350 ml. in all, each extract being cooled to room temperature before filtration. The insoluble residue P8 weighed 7·80 g., the soluble fraction P9 12·2 g. The analyses of the lead compounds P6 and P8, together with the lecithin-kephalin fraction P9, were as follows:

	Yield g.	C %	H %	N %	P %	Pb %	Ash %
P6	0·57	52·02	7·74	0·12	3·25	21·6	30·7
P8	7·80	46·79	7·06	0·13	3·69	23·3	34·8
P9	12·20	62·18	10·11	1·30	2·45	—	2·2

The analytical figures for fractions P6 and P8 are in fair agreement with those required by lead phosphatidate, although these products are not pure. Our experience bears out that of Smith and Chibnall [1932]. These workers comment on the difficulty of obtaining reproducible figures for carbon and hydrogen on similar lead salts. Thus their crude lead phosphatidate preparation gave C, 50·3; 48·2 %; and on purification by precipitation by alcohol from a solution in ether these salts contained 47·0 to 48·3 % of carbon, as against their theoretical content of 51·2 %. Hence not too much reliance can be placed on these carbon figures, which are usually some units below the theoretical value. Our two fractions contain also about 0·1 % N, which we failed to remove in spite of various treatments attempted for this purpose. This same difficulty was experienced by Chibnall and Channon [1927] and Channon and Chibnall [1927], who were unable to remove the 0·11 % N from the free phosphatidic acid prepared from cabbage leaves. Further evidence that these products are mixtures was obtained in the preparation of phosphatidic acid from the lead salt. 0·39 g. P6 was suspended in 40 ml. of hot benzene and decomposed with hydrogen sulphide. Considerable difficulty was encountered in dealing with the colloidal solution which resulted. It was finally evaporated to dryness and the residue extracted with acetone. After filtration, the acetone solution was cooled to 0° for 24 hours and again filtered. On evaporation there was obtained 0·284 g. of a viscous oil, which represented 73 % of P6 as against the 78·4 % required by theory. This contained 3·9 % P (theoretical P 4·42 %) and had I.V. 108. Similar difficulties with colloidal solutions were encountered in the decomposition of 1 g. of fraction P8. This was treated similarly and, after standing in the ice-chest, the final acetone solution yielded after filtration 0·70 g. (P, 4·25 %; N, 0·15 %), whereas the theoretical requirement is 0·77 g. Further the mol. wt. of the product 718, determined by titration in alcohol, shows the presence of non-acidic impurities. Similar findings were recorded by Smith and Chibnall [1932] whose product had mol. wt. 746. This was confirmed by hydrolysis of 1·09 g. of P8 by boiling for 20 hours with 2·5 *N* hydrochloric acid. The yield of fatty acids so obtained, 0·542 g., was 49·6 % of P8 which is no more than 79·8 % of theory, an experience again similar to that of Smith and Chibnall, who obtained 77 % from the lead phosphatidate prepared from grass. These fatty acids had I.V. 107, and contained 24·4 % of saturated and 75·6 % of unsaturated acids determined by the Twitchell method. This I.V. is greater than that of the acids from the lecithin-kephalin fraction, 90·2, but no significance can be attached to this difference, because considerable oxidation of the acids must have taken place during the two years throughout which this work was being carried out, since the I.V. of the acids of the starting material was 120. It seems to us not improbable that the low yields of fatty acids from the lead salts is due in part to oxidation of the more unsaturated constituents and their consequent insolubility in light petroleum on liberation by hydrolysis. This might, in part, also explain the low carbon figures which are generally obtained in these preparations.

The lecithin-kephalin fraction P9.

The N : P ratio in this fraction was 1 : 0·9, and the low nitrogen and phosphorus values showed that not more than two-thirds of this fraction could be lecithin. The excess of nitrogen over phosphorus, together with the demonstration of the presence of carbohydrate, which is mentioned below, show that, in spite of the treatment by emulsification in water twice and shaking the ethereal solution with aqueous acid, this fraction still contains much non-lipoid material.

Calculation suggests that about 20 % of this fraction (3·1 g.) is not lipoid, which shows that the impurities still present after the double emulsification process, about 15 % of P3, have accumulated in this fraction. The difficulty of removing carbohydrates from plant phosphatide preparations has been encountered by many workers [Levene and Rolf, 1925; Schulze and Winterstein, 1903–04; Winterstein and Smolenski, 1908–09; Smolenski, 1908–09; Trier, 1913]. No attempt could be made to isolate lecithin or kephalin from so small an amount of material, and we accordingly contented ourselves with carrying out various experiments, designed to throw further light on the composition of this fraction.

(*a*) *Presence of carbohydrate.* Acid hydrolysis sufficiently prolonged to ensure the complete breakdown of the phosphatide resulted in the presence in the aqueous filtrate of reducing substances to the extent of 3 % when calculated as glucose. This contamination of plant phosphatide preparations with sugars is a general phenomenon, and Smith and Chibnall [1932] point out that the conditions used for acid hydrolysis of phosphatides are such as to reduce, possibly seriously, the yield of reducing sugar found. Hence this figure of 3 % is probably considerably too low and carbohydrates may thus account for a considerable part of the non-phosphatide material.

(*b*) *Fatty acid content.* By alkaline hydrolysis with alcoholic potassium hydroxide, the fraction was found to contain 6·8 % of unsaponifiable matter and 57·39 % of fatty acids. If present as lecithin, the phosphorus content would yield about 45 % of fatty acids on hydrolysis. The excess 12·0 % of fatty acids (about 1·5 g.) may have been produced by slight hydrolysis during the very many fractionation processes. The fatty acids had I.V. 90·2 and yielded by the Twitchell process 58 % of unsaturated acids and 42 % of saturated acids.

(*c*) *Amino-N content.* Determinations of total and amino-N on aliquot parts of the aqueous filtrate from an acid hydrolysis of 2 g. were carried out. The amino-N 0·30 % represented 25·7 % of the total N present in the hydrolysate, or 46·6 mg. which was the amount present in the original material P1. The remainder of this hydrolysate was used for the base separation.

(*d*) *Isolation of choline and colamine as gold salts.* The methods used by Smith and Chibnall [1932] were employed for the separation. The aliquot of the hydrolysate employed contained 3·53 mg. of amino-N and yielded 65·3 mg. of colamine chloroaurate, which was 65 % of that likely to be present from the amino-N content. It melted at 190° and contained 48·1 % Au (required 189–191°, Au 49·2 %). The filtrate from the mercuric acetate precipitation of the colamine was made acid and concentrated, and the mercury was removed as sulphide. After evaporation to dryness, the residue was dissolved in alcohol and an excess of alcoholic mercuric chloride added. The choline mercurichloride which crystallised was obtained in poor yield in very impure state. The mercury was therefore removed by means of hydrogen sulphide and after concentration of the filtrate a slight excess of gold chloride was added. The 10·29 mg. of non-amino-N present in the original aliquot gave 292 mg. of gold salt (M.P. 240–241°; Au 42·9 %). This product was equally impure, for choline chloroaurate melts at 269° and contains 44·5 % Au. From several preparations of the choline fraction of the phosphatide base, the highest melting-point for the gold salt was 248–249°.

Investigation of the inorganic constituents.

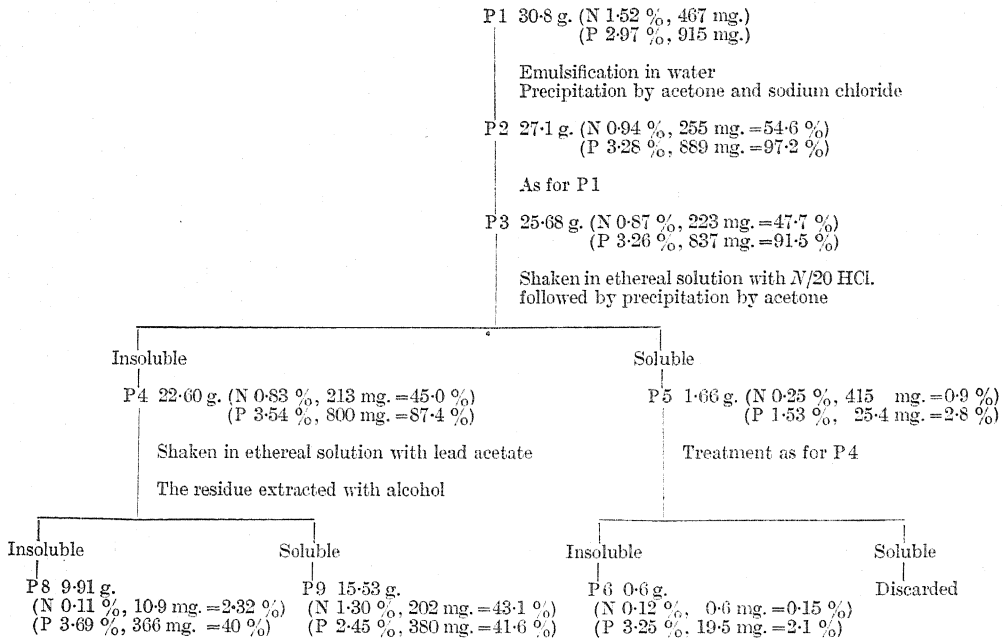
5·19 g. P1 were hydrolysed by boiling for 15 hours with *N* HCl. The fatty acids were removed by extraction with ether. The combined aqueous acid washings were concentrated to small volume and contaminating organic matter

removed by oxidising with nitric acid and perhydrol. Estimations of calcium as oxalate, magnesium colorimetrically and potassium as sulphate on suitable aliquot parts of the solution gave: Ca, 23·4 mg.; Mg, 28·4 mg.; K, 11·1 mg. The amounts of the metals found correspond to 59·22 mg. P, when calculated in ratios Ca/P, Mg/P, K/P, of 1, 1 and 2 respectively. If these metals were originally present as phosphatidates, the amounts in the 5·19 g. of P1 would be Ca phosphatidate, 0·432 g. (8·3 %); Mg phosphatidate, 0·8553 g. (16·5 %); K phosphatidate, 0·1103 g. (2·12 %). In the absence of other contaminating inorganic constituents these percentages of salts of phosphatidic acid would cause the original preparation to give an ash (as pyrophosphates) of 4·41 % (found 4·27 %).

Discussion.

For ease of discussion of the results, a summary of the fractionation is given in outline below. The percentages of N and P in each fraction are recorded, together with the total N and P contents, and these are expressed as a percentage of the corresponding figures on the original material. The weights of fractions have been corrected for the aliquots used for analysis.

Diagram 1. Balance sheet showing the corrected weights, nitrogen and phosphorus percentages and percentage recovery.



In this discussion of the relative amounts of the various constituents present, the calculations will refer to the phosphorus content and not to the gross weight of the individual substances, as it appears more informative to discuss the distribution of the phosphatides on this basis. The diagram shows that the total lead phosphatidate isolated in fractions P6 and P8 contains 42·1 % of the total phosphorus originally present. Since choline and colamine were isolated as gold salts from the lecithin-kephalin fraction in amounts which compared favourably with the content of total and amino-N, it is reasonable to regard

the amino-N of this fraction as originating from kephalin, and to assess the remainder of the P after subtracting the kephalin-P as representing lecithin. Fraction P9 thus contains 2·48 g. kephalin and 7·2 g. lecithin, corresponding to 11·3 % and 30·4 % of the P of the original material. It remains to account for the phosphorus in the aqueous mother-liquors resulting from the emulsification of fractions P1 and P2. We made no attempt to investigate these further, save to show that the phosphorus present in them was not in inorganic form. Bearing in mind the impossibility of precipitating lecithin quantitatively by the means adopted, it is probable that the greater part of this phosphorus was present as lecithin. If this be assumed and the possibility that the original material contained substances containing phosphorus other than phosphatides be disregarded, a balance sheet to show the lipin-P distribution may be drawn up. We recognise of course the limited value of attempting to assess the proportions of different phosphatides in a mixture in the light of the limited knowledge of the constituents and the deficient methods of separation at present available, but such a distribution table is of some value in this particular instance, where attempts to maintain all separations as quantitative as possible have been made. On this basis, the total P of the original mixture had the distribution: lecithin, 46·6 %; kephalin, 11·3 %; phosphatidic acid, 42·1 %.

It remains to correlate these findings with the percentage of ash and its composition. It is shown in a preceding section that calculation of the amounts of the Ca, Mg and K in fraction P1 corresponded to a theoretical ash value of 4·41 % as against 4·27 % found if the metals were present as salts of phosphatidic acid. These amounts would account for 1·141 % of the P of P1, or 38·41 % of the total. The excellent agreement between this figure and that derived from the amount of crude phosphatidic acid, 42·1 %, must however be regarded with caution. In the first place the literature shows repeatedly that preparations of pure lecithin almost always give some figure for ash, and in our own experience two such preparations gave varying figures of 1 to 3 %. Our phosphatide preparation P1 with an ash of 4·27 % therefore was giving a relatively low value, and this percentage is no more than is required were its metallic constituents entirely present as phosphatidates. Secondly, it seems rather doubtful if the potassium were present as phosphatidate, for the alkali salts of phosphatidic acid are water-soluble. It must not be overlooked, however, that ethereal solutions of phosphatides will readily dissolve many substances which are insoluble in ether though soluble in water.

Previous work on the phosphatides of wheat germ has been confined to determinations of ether-soluble P or the recognition of the presence of lecithin from a study of the hydrolysis products. In regard to these and other findings, reference may be made to the work of Schulze and Steiger [1889], Winterstein and Hiestand [1907–08], Schulze [1908], Andrews and Bailey [1932], Sullivan and Near [1933], Knowles and Watkin [1932].

The function which salts of phosphatidic acid are playing in the plant cannot be assessed on the present limited evidence. In leaves, Channon and Chibnall found calcium phosphatidate to be the only phosphatide present in the case of the cabbage. Smith and Chibnall [1932], who encountered very great difficulty in extracting the phosphatides of grass, were finally compelled to use grass dried for 8 minutes at 200°. This material contained many phosphatide decomposition products but the investigations showed that in this leaf lecithin and kephalin were also present. Jordan and Chibnall [1933] obtained 0·4 g. of crude phosphatide from the embryo axes of 5000 seeds of the runner bean and, so far as could be achieved on this very limited amount of material, the results

suggested that the major part of the mixture consisted of a salt of phosphatidic acid, together with lecithin-kephalin in the proportions of 1:2. Our results on wheat germ give a corresponding ratio of 1:1·5. These authors found that on germination the disappearance of the cotyledon phosphatides was much more rapid than the appearance of phosphatides in the growing organs and hence they deduced that seed phosphatides must function chiefly as reserve food.

Summary.

1. Before fractionation of a preparation of the phosphatides of wheat germ, investigations were made of the best methods for extraction of the germ and of precipitation of the phosphatide from the extract.

2. Analyses of the oil prepared from seven samples of germ are recorded. The phosphatide fractions from these extracts were analysed for their contents of nitrogen, phosphorus and ash, together with the yields of fatty acids obtained by hydrolysis.

3. The deductions made from these analyses were confirmed by a detailed investigation of the phosphatide fraction prepared from 9·5 kg. of wheat germ. This fractionation showed the presence of phosphatidic acid (as calcium, magnesium and potassium salts), lecithin and kephalin. These substances occur in the approximate proportions of 4:4:1 when referred to the phosphorus content; at least 42 % of the total phosphatide being present as phosphatidic acid.

4. A method of working ethereal solutions of phosphatides with aqueous solutions, which largely overcomes the persistent emulsions usually resulting, is described.

The expenses of this research were defrayed in part by a grant from the Government Grants Committee of the Royal Society to whom we wish to express our gratidude.

We wish to record our thanks to Messrs Bemax, Ltd., who supplied the fresh wheat germ.

REFERENCES.

Andrews and Bailey (1932). *Ind. Eng. Chem.* **24**, 80.
Channon and Chibnall (1927). *Biochem. J.* **21**, 1112.
Chibnall and Channon (1927). *Biochem. J.* **21**, 233.
Jordan and Chibnall (1933). *Ann. Bot.* **185**, 163.
Knowles and Watkin (1932). *J. Agric. Sci.* **22**, 755.
Levene and Rolf (1925). *J. Biol. Chem.* **62**, 759.
MacLean (1911). *Biochem. J.* **6**, 346.
Schulze (1908). *Z. physiol. Chem.* **55**, 338.
—— and Steiger (1889). *Z. physiol. Chem.* **13**, 364.
—— and Winterstein (1903–04). *Z. physiol. Chem.* **40**, 101.
Smith and Chibnall (1932). *Biochem. J.* **26**, 1345.
Smolenski (1908–09). *Z. physiol. Chem.* **58**, 522.
Sullivan and Near (1933). *Ind. Eng. Chem.* **25**, 100.
Trier (1912). Ueber einfache Pflanzenbasen und ihre Beziehungen zum Aufbau der Eiweissstoffe und Lecithine. (Berlin.)
—— (1913). *Z. physiol. Chem.* **86**, 1.
Winterstein and Hiestand (1907–08). *Z. physiol. Chem.* **54**, 288.
—— and Smolenski (1908–09). *Z. physiol. Chem.* **58**, 506.

CXXII. THE RELATION OF THE GROWTH RESPONSE TO DOSAGE OF VITAMIN A.

CONFIRMATION OF A CURVE RELATING RESPONSE TO DOSE OF VITAMIN A GIVEN.

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Coward *et al.* [1930] published a curve which related the average increase in weight in 5 weeks of groups of rats which, having ceased to grow on a diet deficient in vitamin A, had then been given graded doses of a certain sample of cod-liver oil. Each group consisted of about 30 rats (nearly equal numbers of males and females) and all the rats of any one group were given the same daily dose of cod-liver oil for 5 weeks. The different groups of rats were given daily doses of 0·25, 1·0, 1·5, 2·5, 7·5 and 20·0 mg. cod-liver oil respectively. The curve through the points obtained by plotting the mean increases in weight of the different groups against the dose of cod-liver oil given was a remarkably smooth one and apparently logarithmic in character. By plotting the mean increases in weight against the logs of the doses given, the points were found to be nearly in a straight line. The best straight line through these points was calculated, $y=12{\cdot}1+40{\cdot}2x$; in the form $y=12{\cdot}1+40{\cdot}2\log x$ it applied to the curve itself. Thus it follows that in a comparison of two substances the relative potency of the doses tested is not the ratio of the mean increases in weight of the groups of rats used; it is the ratio of the abscissae corresponding to the mean increases in weight in the curve relating mean increase in weight to dose of vitamin A given; for example if, in a simultaneous test of two samples of cod-liver oil, 1 mg. of one sample given daily for 5 weeks produced a mean increase in weight of 16 g. in a group of 5 male and 5 female rats, and 1 mg. of another sample of cod-liver oil produced a mean increase in weight of 8 g. in a similar group of rats, the ratio of the potencies of the two samples would not be 16:8=2 (the ratio of the mean increases in weight) but 1·25:0·79=1·58 the ratio of the abscissae corresponding to the mean increases in weight as obtained from Coward's curve of response.

Use of this curve for comparison of two substances of different kinds.

The curve of response is needed for interpreting the mean increases in weight of two groups of rats which have been given doses of very different substances (*e.g.* cod-liver oil and, say, milk powder). It is therefore necessary to show that it holds good (*i.e.* measures only vitamin A), no matter what other factors may be unavoidably given to the rats in either or both of the doses of substance under test. That a curve of response may be unreliable and even useless has been shown by Coward *et al.* [1933] in work on the estimation of vitamin B_1. A rather shallow curve of response was obtained when vitamin B_2 *etc.* was

supplied as 8 % yeast autoclaved for 6 hours at 15 lbs. pressure in the basal diet, and the results from pairs of doses of such substances as wheat germ, dried yeast and a certain food product could not be interpreted by it. The result from the higher dose of each substance was much higher than any point on the curve. However, when a fresh curve of response was constructed from results obtained with 20 % autoclaved yeast in the basal diet, a steeper curve of response was obtained than before and the results which could not be interpreted by the first curve could be interpreted by the second. Obviously there had been insufficiency of some factor (B_2?) in the first basal diet so that the full effect of the doses of vitamin B_1 alone could not be produced, but the substances tested (wheat germ, dried yeast, *etc.*) had apparently made up the deficiency.

This experience and a similar experience in the construction of curves of response relating increase in weight and dose of vitamin D given [Coward *et al.*, 1932] made us examine all the results of our vitamin A tests in which we had tested two or more doses of any one substance. The results indicate that our curve of response for vitamin A holds good, no matter what may be the nature of the substance tested. This, in turn, indicates that the basal diet used in our vitamin A tests contains enough of all substances which are necessary for growth except vitamin A.

The examination of results from two doses of any one substance.

In each experiment in which we have tested two or more different doses of one substance for vitamin A, we have determined the apparent potencies of the doses tested by finding from the curve of response the abscissae corresponding to the mean increases in weight of the rats used for each dose. These give the apparent ratio of the doses, and by comparison of the apparent ratio with the known ratio some idea of the reliability of the curve of response may be obtained.

In practice we have seldom used the curve of response already described in this paper, for it requires an equal number of male and female rats in each group of animals used. In 1931 we separated the results from the male and female rats in the groups used for constructing the curve of response, and constructed other curves of response for male and female rats separately. The curves were $y = 11{\cdot}3 + 50{\cdot}3 \log x$ for the bucks and $y = 12{\cdot}4 + 27{\cdot}4 \log x$ for the does. Later, with more information from our tests, these equations became $y = 13{\cdot}4 + 45{\cdot}81 \log x$ (bucks) and $y = 12{\cdot}5 + 27{\cdot}0 \log x$ (does). Thus we now average separately the increases in weight of the bucks and does given any one dose, find the corresponding abscissae from their respective curves of response and find the weighted mean of these abscissae. This can then be compared with the figure similarly obtained from a group of rats on another dose.

In Table I are collected the results of all experiments in which two or more doses of a substance have been tested for vitamin A. In the last two columns are stated (*a*) the known ratio of the doses, and (*b*) the apparent ratio of the doses determined as just described. The average abscissae have been included in the table to show how wide a range of doses the curve may cover.

The results show very good agreement between the known ratio of the doses tested and the apparent ratio of the doses. All the tests but one (dried milk (*c*)) show better agreement than might be expected from the known error of the test. This is the more satisfactory when the varied nature of the products tested is realised. The products include butter and margarine (very good agreement), two samples of cod-liver oil (less good agreement, one rather high and the other rather low), a fish-meal (very good agreement), four samples of dried milk (two in good agreement, one too high and one too low and therefore not disturbing),

Table I. *Comparison between the known ratio of pairs of doses of different substances tested for vitamin A and the apparent ratio of the doses as determined from the abscissae of the curve of response corresponding to the mean increases in weight found in the tests.*

When the substance was given as a percentage of the diet, records were made daily of the amount of food eaten, and from these were calculated the average amount of test substance eaten per day throughout the experiment.

Substance	Dose	Weighted mean of abscissae for male and female rats	Known ratio of doses	Apparent ratio of doses, *i.e.* ratio of abscissae
Dairy butter	0·05 g. 0·1	1·34 3·36	2 : 1	2·5 : 1
Vitaminised margarine	0·05 g. 0·1	1·66 3·4	2 : 1	2 : 1
Cod-liver oil C	1 mg. 3 mg.	0·41 1·77	3 : 1	4·3 : 1
Cod-liver oil G	1 mg. 3 mg.	0·55 1·09	3 : 1	2 : 1
Fish-meal	2·5 % 25·0 %	2·37 25·6	10 : 1	10·8 : 1
(*a*) Dried milk (0·4 %) (2·0 %)	0·022 g. 0·15	0·42 5·05	7 : 1	12 : 1
(*b*) ,, (0·2 %) (2·0 %)	0·013 g. 0·155 g.	0·84 7·2	12 : 1	8·5 : 1
(*c*) ,,	0·1 g. 0·88 g.	3·2 12·0	9 : 1	3·8 : 1
(*d*) ,,	0·005 g. 0·04 g.	0·25 1·87	8 : 1	7·5 : 1
(*a*) Vitamin prep. 2 % 5 %	0·1 g. 0·36 g.	0·71 1·99	3·6 : 1	2·8 : 1
(*b*) ,,	0·01 g. 0·02 g.	1·3 3·4	2 : 1	2·6 : 1
Wheat embryo prep. 10 % 20 % 40 %	0·71 g. 1·56 g. 3·2 g.	0·64 1·74 3·86	4·5 : 2·2 : 1	6 : 2·7 : 1

two vitamin preparations (good agreement), a wheat embryo preparation (fairly good agreement). It is perhaps more satisfactory still when it is realised that some of the best results were obtained in tests where the ratios of the doses were greatest, *viz.* 10:1 and 8:1.

We therefore feel some confidence in using these curves of response for comparing mean increases in weight of groups of animals as treated in our vitamin A tests. The basal diet in use in vitamin A tests in this laboratory contains at least enough of each of the factors necessary for growth for the amount of vitamin A in the dose tested to exert its full effect.

The possible use of these curves in other laboratories.

Considering the influence of partial deficiencies of certain factors in the basal diet on the slopes of curves of response, it would be unwise to adopt the curves described in this paper in another laboratory without confirming them. The confirmation would not need as many rats as were used in the construction of these curves. Probably 10 male and 10 female rats on each of 3 doses, fairly widely distributed; for example 0·5, 2·0, 8 mg. of an average sample of cod-liver oil would suffice for this purpose.

Discussion.

We can find only one other report of the influence of the basal diet on the slope of the curve of response to graded doses of a vitamin. Sherman and Munsell [1925] published a series of composite growth curves of groups of rats which had been depleted of reserves of vitamin A and had then been given graded doses of tomato juice. Later Sherman and Batchelder [1931] published a series of composite growth curves of rats similarly prepared and then given graded doses of dried milk powder. They stated that "milk powder experiments showed a more accentuated response than doses of tomato to increasing levels of feeding." It is a simple matter to construct curves of response for 1, 2, 3, 4, 5, 6, 7 or 8 weeks' feeding from the figures of their two papers. We constructed the curves of response for 5 weeks' feeding, partly for comparison with our own curve, and partly because Sherman and Batchelder suspected that the rats of the most rapidly growing group suffered from a shortage of the "Coward factor" in the last week or two of the test. We could construct only one curve of response for each test, and not two separate ones for male and female rats respectively as the details for male and female rats were not given separately. Each group, however, consisted of approximately equal numbers of bucks and does. The curve of response to graded doses of dried milk powder was very much steeper than that to graded doses of tomato juice, and Sherman and Batchelder say "the less accentuated response to increasing levels of feeding of the tomato may have been because this was a less adequate source than milk of some factor or factors whose influence upon growth was not fully realised at the time of the earlier experiments, or possibly that, notwithstanding the general equivalence of the vitamin A of animal origin, and its precursor of vegetable origin, there may still be a difference in the degree of readiness and completeness of utilisation of the two forms." It is of interest to note that the basal diet used in Sherman's tomato experiment contained only 5 % of dried brewer's yeast, whereas the diet used in the milk experiment contained 10 % dried brewer's yeast. The greater "spread" of the results of the tests of graded doses of dried milk may have been due to the larger supply of some factor in the dried yeast or to some factor other than vitamin A supplied by the milk and not contained in the basal diet. Whichever was the cause, the results demonstrate afresh the necessity of using a basal diet which has been proved to contain sufficient of all factors necessary for growth except the one for which a test is to be made.

Morgan has constructed curves of response relating mean increase in weight in 5 weeks of rats which, having nearly ceased to grow on a diet similar to our vitamin A-free diet, have then been given graded doses of vitamin A (private communication). They are $y_1 = c_1 + 37{\cdot}6 \log x$ (bucks) and $y_2 = c_2 + 26{\cdot}1 \log x$ (does), but as his rats fed on this diet do not cease to grow on the basal diet alone, the slopes of the curves cannot fairly be compared with our own.

The following equations of the curves of response of similar tests for vitamin A, so far available for comparison, are therefore:

(*a*) Sherman's tomato juice curve, $y = 9{\cdot}9 + 22{\cdot}6 \log x$.
(*b*) Sherman's dried milk curve, $y = 5{\cdot}3 + 69{\cdot}8 \log x$.
(*c*) Coward's curve, $y = 12{\cdot}1 + 40{\cdot}2 \log x$.

It appears to be easy to explain the difference in slope of Sherman's two curves on the grounds of insufficiency of some factor in the basal diet, but it is difficult to see how this could be the reason for Coward's curve being less steep than Sherman's steeper one, for it has been shown that Coward's curve holds

good for graded doses of substances containing many different growth factors in addition to vitamin A. Does Sherman's very steep curve of response to graded doses of dried milk powder indicate that when rats have been fed on a diet partially deficient in one factor and wholly in another factor for several weeks and are then given abundance of the former and graded doses of the latter, the response will be greater than if the rats had had abundance of the first factor throughout the experiment? It is impossible to say if this is so from the experiments so far recorded. It can only be emphasised again that if a curve of response is to be used for determining relative potencies of doses, it is of the utmost importance that the basal diet should contain abundance of all factors necessary for growth except the one for which the test is being made.

Summary.

The validity of Coward's curve of response relating mean increase in weight in 5 weeks to dose of vitamin A given has been confirmed by the method of comparing the known ratio of two doses of a substance tested with their apparent ratio as obtained from the abscissae of the curve of response corresponding to the mean increases in weight of the rats given the respective doses.

Twelve substances have been tested in this way. They included butter, vitaminised margarine, fish-meal, cod-liver oil, dried milk, wheat embryo, *etc.*

The agreement between known ratio of doses and apparent ratio of doses left no doubt that the curve was reliable, especially in view of the varied nature of the substances tested and the very large ratio of some of the pairs of doses used (*e.g.* 10:1, 8:1).

The range of doses which can be compared by the use of this curve extends from a dose which is just sufficient to maintain weight of rats which have ceased to grow on a diet deficient in vitamin A, to a dose which is 50 times as great. There is no "flattening" of the curve, and it is therefore concluded that there is no deficiency in the basal diet except vitamin A.

Comparison with curves of response from other laboratories has been made and differences in slopes of curves of response have been discussed.

REFERENCES.

Coward, Burn, Ling and Morgan (1933). *Biochem. J.* **27**, 1719.
—— Key, Dyer and Morgan (1930). *Biochem. J.* **24**, 1952.
—— —— and Morgan (1932). *Biochem. J.* **26**, 1585.
Sherman and Batchelder (1931). *J. Biol. Chem.* **91**, 505.
—— and Munsell (1925). *J. Amer. Chem. Soc.* **47**, 1639.

CXXIII. SIMPLIFICATION OF VITAMIN TESTS.

THE EFFECT OF GIVING DOSES HALF-WEEKLY FOR VITAMIN A TESTS, AND ONCE ONLY FOR VITAMIN D TESTS, INSTEAD OF GIVING DOSES DAILY.

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The most laborious part of the work involved in testing for vitamins is, perhaps, the giving of a daily dose of the standard or of the substance under test to the experimental animals. It occurred to us that a great saving of labour would be effected if the doses could be given at less frequent intervals. We therefore carried out two experiments:

1. On the effect of giving (*a*) daily doses, and (*b*) half-weekly doses in a 5 weeks' test for vitamin A (resumption of growth).

2. On the effect of giving (*a*) daily doses, and (*b*) the whole of the 10 days' dose on the first day of the 10 days' curative period in a vitamin D test.

Both experiments indicated that the giving of doses every day in tests for vitamins A and D is unnecessary; the doses can safely be given at longer intervals than 24 hours, though we have not attempted to determine the limits of these intervals.

Moreover, since adopting the simpler method of dosing, we have obtained enough figures to show that it is at least as accurate as the more laborious method of dosing every day.

We have not extended our investigation to the water-soluble vitamins. It is quite probable that, owing to the nature of their action in the animal body (so far as it is at present understood), the giving of doses at longer intervals than 24 hours would not have the same effect as giving the doses daily.

I. Vitamin A test.

A. *Comparison of effects of giving doses (a) daily and (b) half-weekly.*

The technique employed in this test was that described by Coward *et al.* [1930]. A cod-liver oil, chosen for examination by the two methods of dosing, was diluted with olive oil so that one drop of the dilution (19–21mg.) contained 1 mg. cod-liver oil. Some rats which had ceased to grow on a vitamin A-deficient diet were given one drop of this solution daily, others were given 3 drops on Mondays and 4 drops on Thursdays. The mean increases in weight of the bucks and does were calculated separately for each group (Table I).

Table I. *Mean increase in weight of groups of rats which had become steady in weight and were then given cod-liver oil (a) daily, or (b) half-weekly, the total dose for each rat being 7 mg. per week for 5 weeks.*

	No. of rats		Mean increase in weight in 5 weeks g.	
Method of dosage	♂	♀	♂	♀
Daily	5	6	19·6	14·3
Half-weekly	4	7	20·0	14·0

It is obvious that the method of dosing half-weekly has given the same result as the method of dosing daily.

B. *Validity of this result.*

The possibility that two results in our vitamin A tests although apparently equal, may in fact be different, has been worked out for groups of 10 animals. The larger the number of animals in each group, the greater is the probability that the results are indeed alike.

First were calculated the probabilities that any result with a group of rats would be greater or less than the true result by given amounts; *e.g.* Table II shows the calculation by which it was found that there was a 1/22 chance that 10 male rats would give a result either greater than 153·7 % or less than 65·1 % of the true value and that 10 female rats would give a result either greater than 179·8 % or less than 55·6 % of the true value.

Table II. *Calculation of the probability (1/22 chance) that the result of a vitamin A test (a) based on a group of 10 male rats would be greater than 153·7 or less than 65·1 % of the value found, and (b) based on a group of 10 female rats, would be greater than 179·8 or less than 55·6 % of the value found.*

	Bucks	Does
Standard deviation of the increase in 5 weeks of the rats of this colony in vitamin A tests	14·72	11·02
Curve of response relating mean increase in weight in 5 weeks to dose of vitamin A	$y = 11{\cdot}3 + 50{\cdot}3 \log x$	$y = 12{\cdot}4 + 27{\cdot}4 \log x$
Logarithmic variance of the test (Standard deviation/slope of curve of response)	$\lambda_1 = \frac{14{\cdot}72}{50{\cdot}3} = 0{\cdot}293$	$\lambda_2 = \frac{11{\cdot}02}{27{\cdot}4} = 0{\cdot}402$
Log. var. of mean when 10 rats are used in a test	$\frac{\lambda_1}{\sqrt{10}} = 0{\cdot}093$	$\frac{\lambda_2}{\sqrt{10}} = 0{\cdot}127$
Probability (1/22 chance) that a result with 10 rats would be:	*2·007 × 0·093 = 0·1867 = log 1·5371 greater than 153·7 % or less than 65·1 % of the true value	2·007 × 0·127 = 0·2549 = log 1·7984 greater than 179·8 % or less than 55·6 % of the true value

* Table of x, Fisher, *Statistical Methods for Research Workers*, p. 76.

In a test made with 5 bucks and 5 does, there is a 1/22 chance that the true value is either less than 60·3 % (average of 65·1 and 55·6) or greater than 166·7 (average of 153·7 and 179·8) of the value found. There is a 1/44 chance that it would be one or the other. In a comparison of 2 doses with 2 groups of rats (5 bucks and 5 does in each), there is a 1/44 × 1/44 chance that the true value of one dose is 60·3 % of the found value while the true value of the

other dose is 166·7 % of the found value, *i.e.* there is a 1/1936 chance that one value is really more than 166·7/60·3 = 2·76 times the other. This probability is very small indeed. Other probabilities are shown in Table III.

Table III. *Chances that the true ratio of two doses apparently equal may in fact be greater than 1:1.*

Probability	True ratio	Probability	True ratio
1 in 1936	2·76	1 in 144	2·02
1 in 900	2·57	1 in 36	1·63
1 in 400	2·30	1 in 16	1·41
1 in 256	2·18	1 in 4·4	1·03
1 in 196	2·10		

Thus there is only a 1 in 4·4 chance that either of two results which are apparently equal may be more than 3 % greater than the other. This is much smaller than the "probable error" (1 in 2 chance) which is the probability most often used for describing the accuracy of a biological test. There is a 1 in 16 chance of one result being more than 40 % greater than the other. This again is a small chance. We, therefore, felt justified in changing from daily doses to half-weekly doses for vitamin A tests.

The calculation just described was based on the assumption that the standard deviation of the increase in weight in 5 weeks of rats given half-weekly doses of vitamin A was the same as that of rats given daily doses of vitamin A. We have not enough figures to calculate the standard deviation of the increase in weight of rats given doses half-weekly for 5 weeks, but since adopting this method of dosing we have gained a certain amount of information from which we have calculated the standard deviation of the increase in weight of rats given doses half-weekly for 3 weeks. This figure can be compared with the standard deviation of the increase in weight of rats given doses daily for 3 weeks (Table IV).

Table IV. *Standard deviation of the increase in weight in 3 weeks of rats which have been given doses of vitamin A (a) half-weekly, (b) daily.*

Doses given		No. of tests	No. of rats	Σd^2	Average variance	Standard deviation
Half-weekly	Bucks	78	326	41,936	169·8	13·03
	Does	83	430	30,958	89·22	9·45
Daily	Bucks	184	1014	118,764	143·09	11·96
	Does	194	1187	95,616	96·29	9·81

Thus the standard deviation of the increase in weight in 3 weeks was slightly higher for the bucks and slightly lower for the does which were given doses of vitamin A half-weekly, than for the corresponding rats which were given doses daily. It was, therefore, reasonable to use the standard deviation of the increase in weight of rats given daily doses for 5 weeks for calculations relating to the increase in weight of rats given half-weekly doses for 5 weeks.

II. Vitamin D test.

A. *Comparison of effects of giving (a) one dose at the beginning of the 10 days' curative period, and (b) 1/10 of that dose daily for 10 days.*

We made a direct comparison of these two methods of dosing first of all by using the standard solution of vitamin D. This showed that the two methods gave the same result, but we made another comparison of the two methods

using cod-liver oil (our oil Z) lest the finding with the standard should not be applicable to cod-liver oil. This experiment also gave the same result by the two methods and we, therefore, felt justified in adopting the simpler method of dosing in all estimations of the vitamin D content of samples of cod-liver oil.

(*a*) *Standard solution of vitamin D.* 19 rats from 8 different litters (approximately half of each litter) which had received a rachitogenic diet (Steenbock's 2965) for $3\frac{1}{2}$ weeks were each given 5 units of the International Standard solution of vitamin D in one drop (20 mg.) of solution. They were given the basal diet without addition for the following 10 days. 20 rats, the other halves of the same litters, having as nearly as possible the same initial weights (*i.e.* paired), were given 0·5 unit standard vitamin D in 20 mg. solution daily. Ten days after dosing began, all the animals were killed and their ulnae and radii were examined by the method in general use in this laboratory [Dyer, 1931; Key and Morgan, 1932]. The average degree of healing of the 19 rats which received 5 units of vitamin D in one dose was 3·00 and the average degree of healing of the 20 rats which received 0·5 unit daily was 3·20. Thus the two methods gave almost the same result.

(*b*) *Cod-liver oil.* 10 rats from 3 litters (half of each litter) were each given 20 mg. cod-liver oil in one dose on the first day of the curative period, and 10 rats, the other halves of the same litters, were each given 2 mg. of the same cod-liver oil for 10 days. The average degree of healing, determined as before, was found to be 2·0 for the animals receiving one 20 mg. dose and 1·7 for those receiving 2 mg. daily. Thus the two methods of dosing again gave almost the same result (Table V). Moreover, the method, which gave a slightly higher result in one experiment, gave a slightly lower result in the other. It, therefore, seemed justifiable to conclude that the two methods gave equal results.

(*c*) *Other substances.* Most substances other than liver oils and vitamin D concentrates contain so little vitamin D that large doses of the substance have to be given in order to produce an effect. Thus butters containing from about 0·5 to 2·0 units per g. have to be tested in doses of 0·5 g. per day. This is about as much butter as can be conveniently given to a rat at one time; hence we have continued to give doses of butters and of most other substances every day as before.

Table V. *Comparison of the effects of giving (a) the whole dose at the beginning of the 10 days' curative period, and (b) a daily dose for 10 days' curative period.*

	Average healing in groups of rats after	
	(*a*) Whole dose at beginning of 10 days' curative period	(*b*) Daily dose for 10 days' curative period
Irradiated ergosterol (5 units altogether)	3·0	3·2
Cod-liver oil (20 mg. altogether)	2·0	1·7

B. *Comparison of the two methods of dosing in vitamin D tests with regard to the accuracy obtainable in the result.*

When we began to give the whole of the 10 days' dose of oil or standard at the beginning of the curative period, we delivered the dose from a micrometer syringe, wiping the nozzle carefully before putting it sideways into the rat's mouth and then wiping it once or twice on the side of the rat's mouth on withdrawal, to make sure that the rat had received the whole of the very small dose.

The volumes of the doses to be delivered were calculated from the average specific gravity of cod-liver oil and of the diluted standard as determined in this laboratory.

When we had used this method for some months we gained the impression that we were obtaining more uniform results than we had previously. A calculation of the standard deviation of the amount of healing, based on observations of 334 rats, gives a standard deviation 0·716. The standard deviation obtained for tests in which doses were given daily was 0·783. The improvement in accuracy is thus very slight; it reduces the probable error of the result, when 20 rats are used in an estimation, from 9 to 8 %. We are, therefore, satisfied that the method of giving the whole dose at the beginning of the 10 days' curative period gives a result which is not less accurate than that obtained by the method of giving doses daily.

We have applied this result to the routine of our vitamin A tests also. Whereas we used to give each rat which was being used for a vitamin A test a dose of 4 units vitamin D in 20 mg. olive oil twice a week, nowadays we give 8 units of vitamin D in 20 mg. olive oil once a week. This is a saving of labour also.

Summary.

The same results were obtained in a vitamin A test on a certain sample of cod-liver oil by giving the required amount either as daily doses or as half-weekly doses.

A statistical calculation showed that the probability that these results are really equal and not only apparently so is very high. There is approximately a 77 % chance that the results are within 3 % of each other. There is a 94 % chance that they are within 41 % of each other.

A further calculation of the standard deviation of the increase in weight during the test, since this method of dosing was adopted in this laboratory, shows that the method gives results of approximately the same degree of accuracy as did the former method. The standard deviation of the results from the bucks was slightly greater, that from the does slightly less, than the corresponding figures obtained by the other method of dosing.

Practically equal results were also obtained in two vitamin D experiments ((*a*) using a certain sample of cod-liver oil, and (*b*) using the International Standard for vitamin D) when the whole dose was given at the beginning of the 10 days' curative period and when the amount was divided into 10 daily doses. The former of these methods of dosing appeared by calculation to be slightly more accurate than the latter.

A great saving of labour in tests for vitamins A and D can be safely effected by giving doses at less frequent intervals than the usual one of 24 hours. There is no evidence yet that this result is applicable to tests on the water-soluble vitamins.

REFERENCES.

Coward, Key, Dyer and Morgan (1930). *Biochem. J.* **24**, 1952.
Dyer (1931). *Quart. J. Pharm. Pharmacol.* **4**, 503.
Key and Morgan (1932). *Biochem. J.* **26**, 196.

CXXIV. THE INFLUENCE OF THE SOLVENT ON THE VITAMIN A ACTIVITY OF (*a*) CAROTENE AND (*b*) COD-LIVER OIL.

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In June, 1931, the Permanent Commission on Biological Standardisation of the Health Organisation of the League of Nations recommended [1931] the adoption of a particular sample of carotene as a provisional standard of reference for the estimation of vitamin A. This was to be prepared from the pooled contributions of five workers from different laboratories by the National Institute for Medical Research, London, where it would be kept and issued as required. The unit of vitamin A suggested was the vitamin A activity of 1γ (0·001 mg.) of this particular sample of carotene.

The standard of reference for vitamin A was adopted provisionally for two years, for it was not known how long carotene would retain its growth-promoting potency. As soon as the standard became available, workers in many laboratories began to use it and, from informal comparisons of results, it early became evident that widely different responses to the same dose of standard were being obtained in different laboratories. It was difficult to believe that the differences in response were entirely due to differences in basal diets of the rats, and it seemed most probable that the cause of the differences would be found in the different oils used for dissolving the carotene for administration to the rats. It was, therefore, thought desirable to make direct comparisons of different oils.

I. The influence of the solvent on the vitamin A activity of carotene.

A. *First comparison of oils as solvents for carotene.*

In the first comparison the standard carotene was dissolved in (*a*) hardened cotton-seed oil, (*b*) ethyl laurate (redistilled), (*c*) arachis oil, respectively, so that a daily dose of 5γ carotene could conveniently be given in 0·02 g. oil. In preparing the solutions, one of two methods was adopted; either (*a*) the ampoule containing the standard was weighed before and after washing out with benzene and the loss taken as the weight of carotene, or (*b*) after transferring the solution to a known weight of melted solvent, the benzene was evaporated *in vacuo* until constant weight was attained and the increase in weight recorded as the weight of carotene added. Solvent was then added to produce the required dilution. The solutions were distributed in amber-coloured glass ampoules which were twice evacuated, refilled with dry nitrogen and immediately sealed. The tests were carried out by our usual method of giving 3 doses on Tuesdays and

4 on Fridays [Coward and Key, 1934]. A fresh ampoule of solution was opened each day on which doses were given. The results of this comparison are seen in Table I. All ampoules of solutions were kept at 0°.

Table I. *Comparison of (a) hardened cotton-seed oil, (b) ethyl laurate and (c) arachis oil as solvents for carotene for use in vitamin tests.*

Dose: 5γ carotene (International Standard) in 0·02 g. oil per day.

Solvent	No. of rats in test		No. of rats surviving		Mean increase in wt. in 5 weeks g.		Abscissae of Coward's curve of response corresponding to mean increase in wt.		Weighted mean of abscissae, *i.e.* relative potency of doses
	♂	♀	♂	♀	♂	♀	♂	♀	
Hardened cotton-seed oil	7	7	2	6	8·5	17·2	0·88	1·47	1·32
Ethyl laurate	5	6	4	6	9·2	22·0	0·91	2·24	1·71
Arachis oil	5	7	5	7	38·6	40·7	3·49	10·79	7·75

Thus the vitamin A value of 5γ carotene dissolved in arachis oil may be five or six times as great as it is when dissolved in hardened cotton-seed oil or in ethyl laurate.

The three oils (without carotene) were tested on 3 groups of rats, 4 rats in each group, each rat being given a dose of 0·02 g. oil per day (3 doses on Tuesdays and 4 on Fridays). One rat given hardened cotton-seed oil lived for 35 days and was then killed; it was 5 g. less in weight than when first given the oil; the other three died in 11, 19 and 27 days respectively. The rats given ethyl laurate died in 6, 7, 17 and 17 days respectively. The rats given arachis oil died in 14, 17, 21 and 35 days respectively. Thus the superiority of the arachis oil solution of carotene cannot be attributed to the presence of vitamin A in the arachis oil.

Some comparisons were made of the yellow and red values of these solutions (5γ carotene in 0·02 g. oil), (*a*) after the ampoules had been kept unopened in the refrigerator for 11 weeks after sealing, and (*b*) after a further week during which the open ampoule had been exposed to the air and warm temperature of the rat room for 20–30 minutes each day. The result may be seen in Table II. The colours were matched against the glasses of a Lovibond tintometer.

Table II. *Colours of solutions (a) after 11 weeks' storage in nitrogen-filled ampoules in refrigerator, and (b) same solutions after exposure to air and warm temperature of rat room for 20–30 minutes daily for one week.*

	Colour of solution from freshly opened ampoule after storage for 11 weeks		Colour of same solution after exposure to air of rat room	
	Yellow	Red	Yellow	Red
Hardened cotton-seed oil	19·6	7·0	20·3	6·4
Ethyl laurate	23·0	3·9	24·0	4·0
Arachis oil	24·0	5·7	24·0	5·7

Thus the colour of the solution seems to be very little indication of its vitamin A activity, for the solution in the hardened cotton-seed oil was only slightly less yellow and was a deeper red than the solution in arachis oil, and the solution in ethyl laurate had the same yellow value and a somewhat lower

red value than the solution in arachis oil. The exposure to the air and warm temperature of the rat room for 20–30 minutes daily for one week did not reduce the colour values of these solutions.

B. *Second comparison of oils as solvents for carotene.*

The second experiment consisted of a comparison of arachis oil and coconut oil as solvents for the Internationl Standard carotene. A private communication from one worker that the activity of carotene in arachis oil had been found to be much less than in coconut oil made us decide to use a dose of 2γ standard in arachis oil and a dose of 1γ standard in coconut oil for this comparison. One ampoule of the standard was used for each solution and the dilution made by the same method as in the last experiment. Three doses were given to the rats on Tuesdays and 4 on Fridays as before, a fresh ampoule of solution being opened each day (Table III).

Table III. *Comparison between (a) arachis oil and (b) coconut oil (161441) as solvents for the standard carotene.*

Solvent	Dose of standard	No. of rats used in the test ♂	No. of rats used in the test ♀	Mean increase in wt. in 5 weeks g. ♂	Mean increase in wt. in 5 weeks g. ♀	Abscissae of Coward's curve of response corresponding to mean increase in wt. ♂	Abscissae of Coward's curve of response corresponding to mean increase in wt. ♀	Weighted mean of abscissae, *i.e.* relative potency of doses
Arachis oil	2γ	4	5	33·5	23·6	2·75	2·56	2·64
Coconut oil (161441)	1γ	5	5	25·0	12·6	1·79	1·00	1·40

The relative potency of the doses as determined from the abscissae corresponding to the mean increases in weight of the groups of rats in Coward's curve relating increase in weight to dose of vitamin A given is 2·64:1·4 which is almost the ratio of the doses of carotene given, 2:1. Therefore, the activity of the standard in these samples of arachis oil and coconut oil (161441) is the same.

C. *Third comparison of oils as solvents for carotene.*

A third experiment was carried out (*a*) to compare different samples of coconut oil and (*b*) to see whether the addition of quinol to the solution would make any appreciable difference to the activity of the carotene within the duration of the experiment[1]. The British Drug Houses, Ltd.. kindly supplied three samples of coconut oil known to be of different origin. The test was made in the usual way on each of these samples, each one being tested with and without 0·1 % quinol respectively. The solutions were made in the usual way, one ampoule being opened on each day of dosing. The dose was at the rate of 1γ carotene per day (Table IV).

It is evident that the addition of quinol has made no difference to the activity of the carotene dissolved in each of these samples of coconut oil. In two of the samples the activity of the carotene appears to be equal, while in the third it appears to be less than in the other two. In order to determine whether this difference is significant a calculation was made on the basis of the increases in weight of (*a*) the bucks which received each oil, whether with or without quinol, and (*b*) the does which received each oil whether with or without

[1] A comparison suggested by the Accessory Food Factors Committee of the Medical Research Council and Lister Institute and undertaken by three laboratories.

Table IV. *Comparison between three samples of coconut oil, each one with and without 0·1 % quinol respectively, as solvents for carotene.*

Solvent	No. of rats used in test ♂	No. of rats used in test ♀	Mean increase in wt. in 5 weeks g. ♂	Mean increase in wt. in 5 weeks g. ♀	Abscissae of Coward's curve of response corresponding to mean increase in wt. ♂	Abscissae of Coward's curve of response corresponding to mean increase in wt. ♀	Weighted mean of abscissae, *i.e.* relative potency of doses	
C.O. 161441 + quinol	5	7	21·8	21·7	1·62	2·19	1·95	Av. 1·8
C.O. 161441 alone	3	8	16·7	19·3	1·28	1·79	1·65	
C.O. 162398 + quinol	3	7	28·3	17·7	2·18	1·56	1·75	Av. 2·1
C.O. 162398 alone	6	7	30·5	23·3	2·41	2·5	2·46	
C.O. 162750 + quinol	4	8	7·2	15·3	0·83	1·28	1·13	Av. 1·1
C.O. 162750 alone	3	7	−2·7	16·4	0·53	1·40	1·08	

quinol. The difference in mean increase in weight of the groups of bucks receiving oils 162398 and 162750 respectively was found to be significant, P (the probability that a correlation should arise by random sampling from an uncorrelated population) being $<0·01$. When, however, the calculation was made not from the increase in weight of each rat, but from the doses corresponding to the individual increases in weight (found from Coward's curve of response) P was $>0·05$ and $<0·1$. Therefore the difference indicated by the male rats is of doubtful significance. For the female rats P lies between 0·2 and 0·3 as calculated by the former method and by the latter method P is $>0·02$ and $<0·05$. The difference indicated by the female rats is therefore insignificant. The latter method must be accepted as the more accurate as the response (increase in weight) is proportional to the log of the dose given, not to the dose itself. When the results from both male and female rats are calculated as one result by the use of the abscissae of the curve only (not the increase in weight), P becomes very slightly less than 0·02. Thus there is some indication that there is a difference between the two samples of coconut oil but these results cannot be considered to have proved it.

II. The influence of the solvent on the vitamin A activity of cod-liver oil.

When the early experiments indicated that the activity of carotene was influenced by the oil in which it was dissolved, it appeared to be possible that the oil with which a sample of cod-liver oil was diluted for a test would influence the vitamin A activity of the cod-liver oil. This was investigated, using our cod-liver oil Z for the tests.

A. *Comparison of oils as diluents for cod-liver oil.*

The cod-liver oil, Z, was diluted so that the solution contained the daily dose of 1 mg. in 0·02 g. oil ($\pm 0·001$ g.). Fresh dilutions were made once a week during the test. The solvents tested were (*a*) hardened cotton-seed oil (the sample already used for the carotene experiments), (*b*) olive oil (sample bought in the open market) and (*c*) coconut oil (sample 161441 from B.D.H.). A simultaneous test of 2γ carotene (International Standard) in the same sample of coconut oil was also made (Table V).

Thus coconut oil may enhance the apparent vitamin A value of cod-liver oil or, alternatively, hardened cotton-seed oil and olive oil may lessen the apparent

Table V. *Comparison of (a) hardened cotton-seed oil, (b) olive oil and (c) coconut oil (161441) as diluents for cod-liver oil in a vitamin A test; and also a simultaneous test of the International Standard for vitamin A in coconut oil (161441).*

Dose (daily)	Solvent	No. of rats ♂	No. of rats ♀	Mean increase in wt. in 5 weeks g. ♂	Mean increase in wt. in 5 weeks g. ♀	Abscissae of Coward's curve of response corresponding to mean increase in wt. ♂	Abscissae of Coward's curve of response corresponding to mean increase in wt. ♀	Weighted mean of abscissae, *i.e.* relative potency of doses
1 mg. cod-liver oil Z	Hardened cotton-seed oil	3	7	1·0	13·1	0·62	1·06	0·93
,,	Olive oil	4	7	12·5	15·7	1·06	1·32	1·23
,,	Coconut oil (161441)	5	7	17·8	30·9	1·35	4·73	3·32
2γ carotene (Int. St.)	Coconut oil (161441)	5	7	37·6	33·7	3·33	5·99	4·88

vitamin A value of cod-liver oil. The significance of the difference between the results for olive oil and coconut oil is expressed by the value found for P, $<0{\cdot}01$.

A simultaneous test of the coconut oil (161441) on 6 rats 0·02 g. per day resulted in the death of five of the rats within 3–28 days. One rat was alive at the end of 5 weeks and had gained 15 g. Thus the coconut oil cannot be suspected of itself containing vitamin A.

B. *Estimation of the vitamin A potency of a sample of cod-liver oil (Z).*

(*a*) A test of a daily dose of 2γ carotene (International Standard) in the same sample of coconut oil (Table V) was made at the same time as the comparison of different oils as solvents for cod-liver oil, in order to determine directly the value of our cod-liver oil Z. It proved to be 1400 International Units per g.

(*b*) At the time of the test summarised in Table III, a simultaneous test was made of cod-liver oil Z. The daily dose was 1 mg. dissolved in 0·02 ($\pm$ 0·001) g. arachis oil. Fresh dilutions were (as usual) made each week (Table VI). From this comparison, cod-liver oil Z was found to contain 1750 International Units vitamin A per g.

Table VI.

1. *Estimation of the vitamin A content of cod-liver oil Z, the oil and the standard both dissolved in arachis oil.*

2. *Comparison of the vitamin A activity of a sample of β-carotene with that of the International Standard, both dissolved in coconut oil (161441).*

Dose	Solvent	No. of rats ♂	No. of rats ♀	Mean increase in wt. in 5 weeks g. ♂	Mean increase in wt. in 5 weeks g. ♀	Abscissae of Coward's curve of response corresponding to mean increase in wt. ♂	Abscissae of Coward's curve of response corresponding to mean increase in wt. ♀	Weighted mean of abscissae, *i.e.* relative potency of doses
1 mg. cod-liver oil Z	Arachis oil	5	5	25·2	24·8	1·82	2·84	2·33
2γ carotene (Inter. St.)	Arachis oil	4	5	33·5	23·6	2·76	2·56	2·64
1γ carotene (Inter. St.)	Coconut oil (161441)	5	5	25·0	12·6	1·79	1·00	1·40
1γ β-carotene	Coconut oil (161441)	5	5	30·0	23·6	2·36	2·56	2·44

There is no significant difference between the results (*a*) and (*b*). Thus the same estimate of the vitamin A potency of a sample of cod-liver oil was obtained by dissolving both oil and standard in either arachis oil or in coconut oil.

C. *Estimation of the vitamin A potency of a sample of β-carotene.*

Part of a sample of β-carotene kindly supplied to the Medical Research Council by Prof. Karrer was compared with the International Standard for vitamin A, both samples of carotene being dissolved in coconut oil, distributed in ampoules and used for feeding as in the previous tests. The result may be seen in Table VI. The β-carotene has about 175 % of the vitamin A potency of the International Standard.

Discussion.

Baumann and Steenbock [1933] have examined the influence of various solvents on carotene with respect to changes in colour intensity of the solutions. By measuring spectrophotometrically the intensity of $E^{1\%}_{1\,\mathrm{cm.}}$ 485 and 460 $m\mu$, they found that, among edible oils, refined cotton-seed oil was outstanding as a stabilising solvent for carotene; esters of lauric and sebacic acids alone were unsatisfactory, but with quinol were as effective as cotton-seed oil. Although reduced by exposure to light, air and high temperature, solutions of carotene in cotton-seed oil were not rendered more stable by exclusion of oxygen or presence of quinol or the antioxidant factor of wheat germ oil. Under the conditions usually obtaining during feeding tests, carotene in cotton-seed oil suffered 26 % loss in the first month. Baumann and Steenbock recommend that the main supply of solution should never be removed from the ice-chest, but that a portion necessary for a week's feeding only should be removed for the short time while doses are given to the animals. Actually this is the procedure that we have always followed in our feeding tests with carotene and vitamin concentrates.

While we have confined our observations almost wholly to the biological activity of our solutions, our one physical comparison of different oils, hardened cotton-seed oil, ethyl laurate and arachis oil, indicated that, after 11 weeks, there was very little difference in the intensities of yellow colour of the solutions, although, during that period, biological tests had shown the solution in arachis oil to be more active than either of the other two solutions. Moreover, exposure of the solutions to the air and warm temperature of the animal room for 20–30 minutes daily for one week did not diminish the colour intensity of the solutions. Thus, while we have demonstrated biologically that different oils used in dissolving carotene for administration to the rats may influence greatly the activity of the carotene, our results with the physical measurements, so far as they go, do not indicate that the intensity of colour of the solutions can be taken as an indication of their vitamin A potency.

Summary.

1. The oil used for solution of the provisional International Standard for vitamin A may greatly influence its biological activity as shown in ordinary vitamin A tests.

2. A solution of the Standard in arachis oil had 5–6 times the potency of solutions in hardened cotton-seed oil and ethyl laurate, though made of the same strength, *w/v*.

3. Solutions of equal strength of the Standard in one sample of arachis oil and in coconut oil (161441) had the same vitamin A potency.

4. Two samples of coconut oil gave solutions of the same potency. A third sample appeared to give a solution of less potency but the result was not conclusive proof of a difference.

5. The oil used to dilute cod-liver oil for dosing may have some influence on the vitamin A potency of cod-liver oil also. Cod-liver oil Z dissolved in coconut oil (161441) was about three times as active as when dissolved in hardened cotton-seed or olive oil. The coconut oil itself (161441) was shown to be without vitamin A activity.

6. When cod-liver oil Z and the Standard were both dissolved in arachis oil for testing, the same estimate of the vitamin A content of oil Z was obtained as when both were dissolved in coconut oil.

7. An estimation of the vitamin A potency of a sample of β-carotene showed it to have about 175 % of the vitamin A potency of the International Standard.

8. The intensities of the yellow colour of different solutions of the Standard did not correspond with the vitamin A activities of the solutions but this comparison was made in only one experiment.

REFERENCES.

Baumann and Steenbock (1933). *J. Biol. Chem.* **101**, 561.

Coward and Key (1934). *Biochem. J.* **28**, 870.

Permanent Commission on Biological Standardisation, Health Organisation of the League of Nations (1931). Official No. C.H. 1056 (1), Geneva.

CXXV. OBSERVATIONS ON THE CHEMISTRY OF LIVER GLYCOGEN.

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(*Received March 12th, 1934.*)

The means of identification of glycogen at the investigator's disposal are of necessity limited by the physical nature of the material; indeed the identity of glycogens prepared from different sources has never been satisfactorily established. Cremer [1894] and Clautriau [1895] found values for the specific rotation of yeast glycogen of +198·9 and +184·5° respectively. Harden and Young [1902; 1912] found values between +191 and +203° for glycogens prepared from oysters, rabbit-liver and yeast; in addition, the colour developed with iodine varied in tint according to the source. Norris [1913] followed the course of hydrolysis of a number of specimens of glycogen by pig pancreatic amylase and observed that his materials were hydrolysed at different rates when the optimum p_H of the reaction was taken into consideration.

The recent work of Donhoffer and Macleod [1932] has suggested that the glycogen in the livers of fasted rabbits may differ in its physiological behaviour from that in the livers of fed animals, and it is of interest to know whether this physiological differentiation is associated with a difference in chemical properties. We have therefore endeavoured by carefully controlled methods to determine satisfactory criteria for a standard type of glycogen derived from the livers of fed rabbits and then to apply our data to specimens obtained under some different physiological conditions and from various sources. This paper is concerned solely with glycogens obtained from livers.

The criteria which we finally adopted were:

(1) Specific rotation in water.

(2) Reduction of the Hagedorn-Jensen ferricyanide reagent.

(3) Colour developed with dilute iodine solution, and opalescence of solution.

(4) Ash content.

(5) Polarimetric observations during the course of hydrolysis by dilute mineral acid.

Glycogen from fed rabbits.

Isolation.

The methods available fall into two types, *viz.* those in which the glycogen is extracted from the tissues by water and those in which the tissues are completely dissolved by strong alkali. Pflüger [1904] and the earlier workers concluded, with the methods then available, that glycogen was not affected by treatment with hot 30 % KOH solution, but this has been recently questioned; for example Haworth and Percival [1932] find that glycogen has a lower reducing power towards hypoiodite than they would infer from the properties of their

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postulated structural formula. They suggest that the reducing groups originally present in the glycogen molecules have been partly oxidised during treatment with strong alkali. We have therefore prepared, by methods of both types, *i.e.* aqueous extraction and alkali-extraction, a number of specimens of glycogen from the livers of fed rabbits, these specimens being subsequently subjected to examination under parallel conditions.

"Aqueous extracted glycogen" was obtained by the following method, which was essentially similar to that described by Carruthers [1931]. The livers, removed whole from three to five decapitated animals, were immediately plunged into about twice their volume of boiling water and the whole was heated to boiling for 5 minutes during which time the tissue was chopped into small pieces. The solid was then ground with sand and boiled with the original liquors for 30 minutes; it was then strained off and twice re-extracted with boiling water for 30 minutes. The united extracts were cooled, 40 % trichloroacetic acid solution was added until the final concentration was 4 %, and the whole was allowed to stand at 0° for 15–18 hours. The precipitated protein was centrifuged off and the glycogen precipitated by alcohol (2·5 vol.). The crude material was twice reprecipitated from water by alcohol in the presence of a little ammonium acetate as recommended by MacDowell [1927], when the glycogen separated as a pure white powder.

The "alkali-extracted glycogen" was obtained by direct alcoholic precipitation of the alkaline tissue hydrolysate from the action of 30 % KOH solution for 3 hours at 100°, using 3 to 5 livers for each specimen. As before, the crude product was twice reprecipitated from water by alcohol in the presence of ammonium acetate, but here the material invariably showed a faint brownish tinge.

As a final process of purification all the specimens were dialysed for 100 hours in running water in the presence of thymol. Dialysis was effected in collodion tubes prepared according to the directions of Wright *et al.* [1928]. The foregoing procedure yielded products which were nitrogen-free and did not reduce Fehling's solution.

In order to investigate the specific rotation, reducing power, *etc.*, the material was dried over calcium chloride at 100°/0·1 mm. until constant weight was attained. This required about 2 hours. No "denaturation" such as was observed by Sayhun and Alsberg [1930] took place during the drying of our specimens.

Table I.

Specimen	$[\alpha]_D^{18°}$ $c=0·5$ $l=1·0$	Reduction (glucose = 100)	
Alkali-extracted			
1	+191·5°	3·30	Iodine coloration (deep brownish red) and opacities were identical
2	199·0	0·24	
3	191·0	1·94	
4	192·7	0·08	
5	193·5	0·15	
		Mean 1·14	
Water-extracted			
6	192·7	0·21	
7	191·0	0·40	
8	191·0	0·37	
9	194·1	0·60	
10	199·0	0·27	
11	195·8	0·09	
		Mean 0·32	

Solutions in water, of concentration approximately 1·3 %, were prepared and portions diluted to 0·5 % for observations on rotation and reduction.

The results are given in Table I.

From the foregoing results it will be seen that the observed specific rotations of our specimens varied between the limits +191·0 and +199·0°, in both types of material. The alkali-extracted specimens showed considerable irregularity in reducing power, the average value being considerably greater than that of those prepared by aqueous extraction.

Hydrolysis of liver glycogen by aqueous acid.

The polarimetric behaviour on hydrolysis by aqueous acid was then investigated. To suitable amounts of the 1·3 % solutions small quantities of concentrated hydrochloric acid were added, sufficient to give a final concentration of 2·4 % HCl. The initial rotation was observed in a 1 dm. tube, and then the solution was heated at 100°. Observations were made at frequent intervals, using a 2 dm. tube; it was noticed that with both types of preparation the solution became clear after about 15 minutes, but while the solutions from the aqueous extracted material, when clear, were absolutely colourless, those of the alkali-extracted preparations showed a distinctly yellow tinge. On plotting rotation-time curves, a striking difference emerged between the curves derived from the two groups of specimens. Whereas the water-extracted material showed a perfectly regular and smooth curve in each case (Fig. 1), the alkali-extracted specimens, without exception, displayed varying, yet considerable, inflections during the first hour of hydrolysis (*e.g.* Fig. 2).

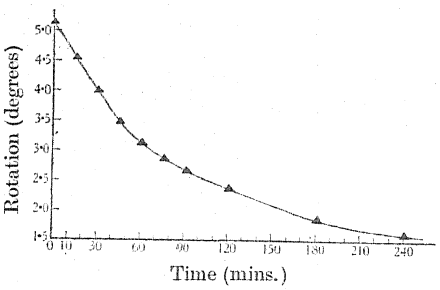

Fig. 1.

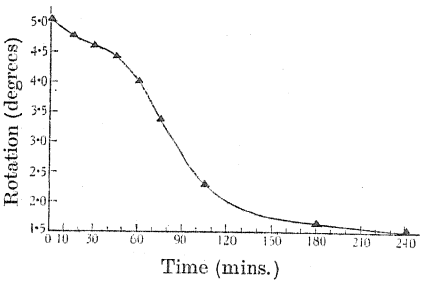

Fig. 2.

Fig. 1. Hydrolysis curve of specimen 8 (prepared by water process). Concentration of glycogen, 1·3 %.

Fig. 2. Hydrolysis curve of specimen 2 (prepared by KOH process). Concentration of glycogen, 1·3 %.

The effect of further treatment with KOH.

Specimens 6 to 10 inclusive (see Table I) were united and reprecipitated from water by alcohol to form specimen A. Amounts of this material, and also quantities of specimens 2 and 3, were heated as 5 % solutions in 30 % KOH for 2 hours, precipitated and dialysed as before.

No change in iodine coloration or opacity could be detected and no loss in material occurred. The results are given in Table II.

Table II.

Specimen	$[\alpha]_D^{18°}$, $c=0{\cdot}5$, $l=1$		Reduction	
	Before KOH	After KOH	Before KOH	After KOH
2	+199·0	+200·0	0·24	0·29
3	191·0	198·4	1·90	0·46
A	195·8	198·1	0·09	0·10

No significant change had occurred in the specific rotation, while the reducing power was considerably diminished where it had been initially large (specimen 3).

As before on hydrolysis by aqueous acid (2) and (3) showed marked inflection of the rotation-time curves, while (A), which had previously given a smooth hydrolysis curve, now exhibited an inflected one (Fig. 2).

The effect of precipitation from water by acetic acid.

Claude Bernard [1877] stated that glycogen could be precipitated from water by the addition of acetic acid, an observation which does not seem to have attracted the attention of later workers.

We have found that glycogen is precipitated quantitatively in 80 % acetic acid solution and have applied this to the estimation of glycogen in tissue, without, however, finding any particular advantage associated with its use in this connection. Nevertheless, precipitation with 80 % acetic acid provides an excellent means of freeing glycogen from the inorganic material with which it is normally associated. Thus we have found that, employing this process, it is possible after four precipitations to obtain glycogen with an exceedingly small ash-content, and in some instances we have prepared ash-free specimens, without having recourse to elaborate methods of electro-dialysis, *etc.* Table III gives some typical results:

Table III.

Specimen	$[\alpha]_D^{18°}$		Reduction		Ash content %	
	Initial	After pptn. by acetic acid	Initial	After pptn.	Initial	After pptn.
(KOH)						
2	+199·0°	+196·7°	0·24	0·11	—	0·195
3	191·0	190·7	1·90	0·06	—	0·224
P	—	196·9	—	0·09	—	0·090
(Water)						
A	195·8	197·0	0·09	0·09	0·036	Nil
11	—	196·5	—	0·09	—	Nil

It will be apparent that this method of purification afforded a series of products displaying closely similar properties; indeed, bearing in mind the nature of glycogen and the difficulties attendant on the determination of its constants, we feel justified in regarding our purified specimens as consisting of a chemical individual. The small residual ash of the alkali-extracted specimens appeared to consist chiefly of silica, presumably introduced during the treatment with strong alkali in a glass vessel.

The effect of ions on the specific rotation.

Neither H nor OH ions, nor the presence of boric acid or borate, were found to affect the rotation of any of our specimens.

The presence of iron in glycogen.

After wet-ashing with pure nitric acid minute traces of iron were detected in most of the purified specimens. In two instances, specimens 8 and 9, the thiocyanate test, which was employed throughout, gave negative results even before the materials had been purified by acetic acid. From this evidence we conclude that iron does not form an essential part of the glycogen complex.

Aqueous acid hydrolysis of glycogen precipitated by acetic acid.

Carrying out polarimetric observations as before, we found that, whether water- or alkali-extracted material was under investigation, in every case the rotation-time curves were identical and uninflected, of the type of Fig. 1. This suggested that the inflections observed in the specimens which had been, at one time or another, treated with strong alkali, originated from the action of the latter substance on the ash soluble in acetic acid. To investigate this view, specimens purified by precipitation with acetic acid were subjected to the action of 30 % KOH as described earlier. On hydrolysis of these materials by aqueous acid no inflection could be detected in the rotation-time curves, thus confirming the above suggestion.

The evidence thus afforded shows that, when free from acid-soluble ash, the rotation-time curves of glycogen obtained by both methods of extraction are identical. We are thus finally able to state that, in so far as we are able to determine, strong alkali does not produce any chemical alteration in the carbohydrate.

GLYCOGEN FROM FASTED RABBITS.

The material was prepared by the aqueous extraction method and was obtained in a yield of 1·83 % of the initial liver weight. In Table IV details are given of the usual constants determined before and after precipitation from acetic acid solution.

Table IV.

	Before reprecipitation	After reprecipitation
$[\alpha]_D^{17°}$	+189·0°	+196·0°
Reducing power	0·26	0·19
Ash content %	0·66	0·13
Iodine coloration	Normal	Normal

On hydrolysis by aqueous acid the material yielded a smooth curve of the type of Fig. 1. These results are thus in no way significantly different from those obtained with the glycogen from fed animals.

RAT-LIVER GLYCOGEN.

The livers of fed rats were extracted with water by the method described in the section on rabbit-liver glycogen. The crude material was not examined but was purified by three precipitations from acetic acid (Table V).

Table V.

$[\alpha]_D^{17°}$	+196·0°
Reducing power	0·08
Ash content %	0·067
Iodine coloration: identical with that given by rabbit-liver glycogen	

This specimen of glycogen was indistinguishable from the polysaccharide obtained from rabbit-liver, with the exception of a possible slightly greater opacity in aqueous solution.

Fish-liver glycogen.

Our material was obtained from fish of the genus *Gadus*, *G. morrhua* (cod), *G. aeglefinus* (haddock) and *G. merlangus* (whiting). The authors are indebted to Mr James Sharp, of the Torry Research Station of the Dept. of Scientific and Industrial Research, for a generous supply of crude material prepared from livers freshly removed from live fish in the following manner. The livers were digested with water at 70° and acetic acid added till the p_H was 4·5. After standing for some time the aqueous layer was run off from the supernatant oil and concentrated to small bulk, and the glycogen was precipitated by the addition of two volumes of 96 % alcohol.

The crude material, as we obtained it, contained much oil and protein. It was therefore exhausted first with boiling spirit, then with carbon tetrachloride. The residue was extracted four times with boiling water, the extract cooled and saturated with picric acid [Pertree and Alsberg, 1929]. The precipitated protein was centrifuged off and the yellow opalescent solution made up to 70 % alcohol by the addition of spirit. The precipitate was collected on a filter and washed with spirit till free from picric acid. It was then dissolved in water and treated with trichloroacetic acid as described above for rabbit-liver glycogen.

Carrying out experiments under conditions parallel with those on the material from rabbit liver we obtained the results given in Table VI.

Table VI.

	Initial	After KOH	After acetic acid
$[\alpha]_D^{17°}$	+196·0°	+194·2°	+196·6°
Reducing power	0·35	0·14	0·16
Ash content %	0·127	0·147	0·028

On hydrolysis by aqueous acid the material behaved exactly as the water-extracted material from rabbit-liver. The reducing power appeared to be slightly higher but we cannot yet say whether this is significant. The coloration produced by iodine was, however, distinctly different, being a deep yellow-brown.

Examination of the products of hydrolysis by aqueous acid.

In order to determine the nature of the units composing the complex of fish-liver glycogen, we decided to employ the process of direct hydrolysis of the polysaccharide by aqueous acid, rather than the now customary procedure of treating the acetate with methyl alcoholic hydrogen chloride and examination of the resulting glucosides [*cf.* Irvine and Hirst, 1924]. The reason for this choice lay in the fact that the yield of acetate obtainable from our material under the mildest conditions [Haworth and Percival, 1932] did not exceed 90 %, thus leaving a not inconsiderable portion of the material unaccounted for. As will be seen our final yield of hydrolysis product consisted of pure glucose amounting to 93·6 % of the starting material.

7·517 g. of purified glycogen were subjected to hydrolysis by 2·5 % aqueous hydrochloric acid. The rotation reached a constant value of +61·3° for the Hg green line after 5 hours. The acid was neutralised with silver carbonate, the solid removed by filtration and colloidal silver eliminated from the filtrate by treatment with hydrogen sulphide and norite. On evaporating the clear solution to

dryness *in vacuo* and drying to constant weight over P_2O_5, 7·818 g. of a light yellow glass were obtained, corresponding with a yield of hexose of 93·6 % of theory.

The crude material was dissolved in spirit and concentrated to a thick syrup under reduced pressure at 50°; the material then crystallised spontaneously. The whole was taken to dryness and recrystallised from 92 % alcohol. Several crops of crystals, amounting to nearly 92 % of the crude material, were collected, all showing identical microscopic appearance (small prisms), M.P. 144–145°. Mixed melting-points with authentic α-glucose (M.P. 144–146°) showed no depression. The united crystals showed in water the typical mutarotation curve of α-glucose.

The mother-liquors were taken to dryness under reduced pressure; the residue again crystallised spontaneously, as before, in small prisms, contaminated by a slight trace of a yellowish syrup. The M.P. was 127°. The rotation in water was considerably lower than that of the pure crystals, but the course of the downward mutarotation was identical with that of glucose. This portion of the investigation is detailed in Table VII.

Table VII.

Material	M.P.	Specific rotation: Initial (extrapolated)	Specific rotation: Final
α-Glucose	144–146°	+110°	+52·70°
Pure crystals (92 %)	144–146°	+110°	+52·59°
Residual material (8 %)	127°	+ 77°	+52·78°

There is thus no evidence of any units other than anhydroglucose being concerned in the structure of glycogen from this source.

Discussion.

From the results of our experiments on the action of strong KOH on highly purified glycogen, prepared in the absence of alkali, we are of the opinion that the carbohydrate is not chemically affected by the treatment. Thus, the suggestion of Haworth and Percival [1932] that alkaline treatment might oxidise a proportion of the terminal reducing groups present in the glycogen chains is in no way substantiated. Indeed, it appears to us that if the suggestions of these workers were correct one would expect to find gluconic acid derivatives among the fission products of their trimethylglycogen.

The observations of previous workers have shown considerable variation in the properties of their preparations of glycogen. We believe that our method of purification, involving precipitation by acetic acid, affords specimens displaying properties as constant as might be expected to be shown by a substance of the physical nature of glycogen. The specific rotations agree closely with one another, the ash contents are low, or zero, when precautions are taken to exclude contamination by silica *etc.*, and, in those specimens examined, the total phosphorus contents were found to be of a low order. The results obtained by previous observers are compared with those of the present authors in Table VIII.

The polarimetric behaviour of our materials on hydrolysis by aqueous acid shows that this process proceeds evenly throughout, there being no evidence of "breaks," which might be expected to occur if the reaction proceeded in different stages. The very closely agreeing figures which we obtained for these observations tend to support the view that our specimens consisted of the same individual

Table VIII. *Liver glycogens.*

Authors	Source	$[\alpha]_D$	Method of purification	% Ash	% P_2O_5
Gatin-Gruzewska [1904]	Dog	+197°	16–18 pptns. from alkali	Nil	—
Harden and Young [1902]	Rabbit	+191°	—	—	—
Norris [1913]	Rabbit	—	Dialysis	0·51	—
,,	Dog	—	,,	0·38	—
Sayhun and Alsberg [1930]	Rabbit	—	Precipitation	0·20	0·032
Haworth *et al.* [1929]	?? (Kahlbaum)	+191°	Electrodialysis	0·37	0·14
Present authors	Rabbit (fed)	+196°	Pptn. by acetic acid	Nil	0·107
,, ,,	Rabbit (fasted)	+196°	,, ,,	0·130	—
,, ,,	Rat	+196°	,, ,,	0·067	—
,, ,,	Fish	+196°	,, ,,	0·028	—

substance. The dissimilarity in iodine colorations developed by mammalian and fish glycogens, although distinct, may not be associated with profound differences in chemical structure.

Further work in this field is at present in progress.

Summary.

1. Criteria for liver glycogen have been determined, *viz.* specific rotation, reducing power, ash content, coloration with iodine and polarimetric behaviour on hydrolysis by aqueous acid.

2. Judged by these criteria, the liver glycogens from the following sources are not significantly different: fasted rabbits, fed rabbits, fed rats and fish of the genus *Gadus*.

3. Glycogen may be obtained in a highly purified, ash-free state by precipitation from water by acetic acid.

4. The chemical properties of highly purified glycogen are not altered by treatment with 30 % KOH at 100°.

The authors desire to express their gratitude to Prof. J. J. R. Macleod for his interest and for the facilities which he provided for this work, and to Sir J. C. Irvine for his generous advice and criticism. Part of the expense of this investigation was defrayed by a grant from the Government Grants Committee of the Royal Society to one of us (F. G. Y.), for which we wish to record our thanks.

REFERENCES.

Bernard (1877). Leçons sur le diabète, p. 303. (Baillière, Paris.)
Carruthers (1931). *Chinese J. Physiol.* **5**, 85.
Clautriau (1895). *Mém. Cour. Acad. Roy. Belg.* 53.
Cremer (1894). *Münch. med. Woch.* **41**, 525.
Donhoffer and Macleod (1932). *Proc. Roy. Soc. Lond.* B **110**, 125.
Gatin-Gruzewska (1904). *Pflüger's Arch.* **102**, 569.
Harden and Young (1902). *J. Chem. Soc.* 1224.
—— —— (1912). *J. Chem. Soc.* 1928.
Haworth and Percival (1932). *J. Chem. Soc.* 2277.
—— Hirst and Webb (1929). *J. Chem. Soc.* 2479.
Irvine and Hirst (1924). *J. Chem. Soc.* 15.
MacDowell (1927). *Proc. Soc. Exp. Biol. Med.* **25**, 85.
Norris (1913). *Biochem. J.* **7**, 26.
Pertree and Alsberg (1929). *J. Biol. Chem.* **82**, 385.
Pflüger (1904). *Pflüger's Arch.* **102**, 305.
Sayhun and Alsberg (1930). *J. Biol. Chem.* **89**, 33.
Wright, Herr and Paul (1928). *J. Biol. Chem.* **80**, 571.

CXXVI. AN ELECTRIC FURNACE FOR MICRO-KJELDAHL DIGESTIONS AND SIMILAR PURPOSES.

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From the National Institute for Research in Dairying, University of Reading.

(*Received March 24th, 1934.*)

In carrying out micro-analyses on blood involving Kjeldahl and other incinerations the need for a suitable furnace was felt, and the apparatus described and illustrated in this note was therefore devised. Electrical heating was preferred to gas on account of its convenience and for another reason mentioned below.

The frame is of angle iron screwed to a teak base. The flasks are supported at an angle of 45° on a perforated sheet of asbestos, their ends projecting for about $\frac{1}{4}$ in. into openings cut in the lead pipe, *A*. A platform made from sheet asbestos supported by strips of angle iron carries two Ferranti 500 watt heating coils, which are held in place by the spring clips, *C*. Each coil serves to heat four flasks. The copper reflectors, *D*, shield the heating elements from draughts and greatly increase the available heat. The platform carrying the heaters is pivoted at the back, and the position of the heating coils relative to the flasks can be altered by raising or lowering the wing nuts, *F*. This arrangement affords a means of adjusting the rate of heating.

The fume absorber, *A*, which slopes slightly from left to right (Fig. 1) is fixed to uprights of spring steel so that its position relative to the flasks can be adjusted by means of the wing nuts, *H*.

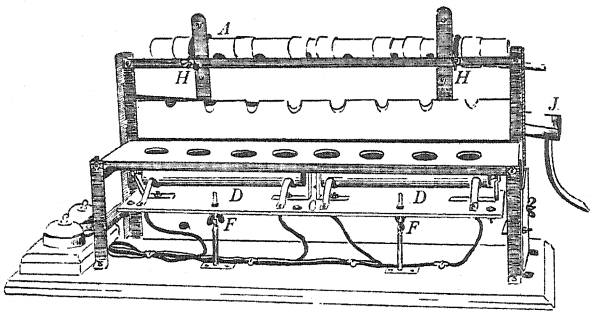

Fig. 1.

The furnace can be safely used in a laboratory without a fume cupboard since when the lead pipe is connected to a good water pump and the unoccupied holes closed by lead slides no fumes escape into the atmosphere.

If the digestion mixture contains an appreciable quantity of water, Kjeldahl flasks are often cracked by water which condenses in the lead pipe and runs down the outside of the flasks. This can be prevented by placing rubber washers

round the flasks at a suitable height so that the condensed liquid drips into the metal trough, *J*, which should be coated with an acid-resisting paint.

In the author's experience micro-Kjeldahl digestions proceed much more smoothly with an electrically heated than with a gas-heated furnace. This may be due to more even heating of the contents of the flasks in the former case. Using the apparatus described for the digestion of whole blood it has been found that when the heating is suitably regulated no bumping takes place. Even with whole milk, a notorious offender in this respect, the amount of bumping is quite insignificant. Moreover, a sample of powdered dried grass, kindly submitted by Dr A. W. Greenhill of the Agricultural Research Station, Jealott's Hill, Berks, in the digestion of which over gas flames excessive bumping had been experienced, was digested on this apparatus with no bumping whatever.

SUMMARY.

An electric furnace suitable for micro-Kjeldahl and similar incinerations is described. The use of an electric furnace provided with a means of regulating the heating eliminates bumping.

I desire to thank N. Gruber for preparing the drawing and P. W. Batten for his assistance in building the furnace.

The apparatus can be obtained from Messrs Callas Sons and May Ltd., 65 Oxford St, Reading.

CXXVII. THE OXIDATION OF THE FATTY DIBASIC ACIDS AND OF LAEVULIC ACID BY HYDROGEN DIOXIDE IN PRESENCE OF A CUPRIC SALT.

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(*Received May 1st, 1934.*)

Considerable quantities of the dibasic acids are formed in the laboratory when the higher fatty acids are oxidised by such reagents as alkaline permanganate or nitric acid; and oxalic, succinic, adipic and suberic acids have been identified. It is however generally accepted that *in vivo* the path of the oxidation of the fatty acids does not proceed by way of the dibasic acids. Verkade and Van der Lee [1934] have however recently shown that when decanoic or undecanoic acid was taken by mouth, the terminal methyl group was oxidised and the corresponding dibasic acid excreted in the urine. In the laboratory, oxidation of the higher fatty acids with hydrogen dioxide is the process which has been supposed most closely to resemble the normal process of oxidation in the body, and recent experiments [Smedley-MacLean and Pearce, 1934] on the oxidation of palmitic acid, in which this process was catalysed by the addition of a cupric salt, have shown that at 90° there is a rapid breakdown, the end-products being succinic, carbonic and the volatile fatty acids. We therefore investigated the oxidation of the dibasic acids with the same reagents. These acids were found to be more readily attacked than the monobasic acids, so that even at 60° no glutaric or adipic acid and only 10 % suberic acid remained unchanged after 1 hour's heating. When palmitic acid was oxidised by excess of hydrogen dioxide in the presence of copper, there was very little evidence of keto-oxidation: the main products were hydroxy-acids, and there was also evidence of a considerable amount of desaturation. With the dibasic acids, definite evidence was obtained of keto-oxidation: thus laevulic acid was formed from adipic acid, a small quantity of acetonylacetone from suberic acid, and acetone was isolated from the products of oxidation of glutaric acid. With suberic acid however there was definite evidence of hydroxylation, the α-hydroxy-derivative and a lactonic acid being isolated.

From all the acids investigated (laevulic, suberic, adipic and glutaric), succinic acid was isolated as an oxidation product, and as succinic acid is itself oxidised under the conditions of the experiment giving a considerable proportion of carbonic and other volatile acids, the succinic acid isolated in these oxidation experiments must represent only a proportion of that actually formed. No evidence of the formation of fumaric, malic or tartaric acid was obtained.

The method of oxidation was that described by Smedley-MacLean and Pearce [1934], the volatile products being separated by steam-distillation.

The proportion of dibasic acids oxidised by a quantity of hydrogen dioxide sufficient to give about 19 % more than the amount of oxygen necessary for the complete oxidation of the acid was considerably augmented by the addition of

Table I. *Oxidation of certain dibasic acids and laevulic acid carried out for 1 hour at 60°.**

	Conditions				In absence of copper					In presence of copper						
Acid oxidised	Weight of acid g.	Total vol. ml.	Acidity (normality)	No. ml. 6 % H_2O_2	CO_2 % exp.†	CO_2 % distillation‡	Reducing volatile acid calc. as % formic	Remaining volatile acids calc. as % acetic	Ether extract g.	CO_2 % exp.†	CO_2 % distillation‡	Reducing volatile acid calc. as % formic	Remaining volatile acids calc. as % acetic	Ether extract g.	Acetone g.	Aldehyde calc. as acetic g.
Succinic	2·5	300	0·0142	96	3·0	1·9	1·6	4·6	2·2	10·6	2·0	2·6	6·8	2·2	—	—
	2·5	300	0·0142	96	1·3	2·1	0·9	22·5	1·7	9·5	4·0	5·0	18·4	1·3	—	—
	2·5	300	0·0140	204	—	—	—	—	—	29·4	13·9	8·1	19·8	>0·3	—	—
	2·5	515	0·0242	350	—	—	—	—	—	45·6	10·1	1·8	14·0	0·6	—	—
Glutaric	2·5	300	0·0142	122	0·6	0·5	0·1	3·5	2·1	26·2	4·8	4·7	16·7	>1·4	—	—
	2·5	515	0·0346	350	—	—	—	—	—	53·3	13·1	2·8	40·2	0·3	—	—
	2·5	515	0·0242	350	—	—	—	—	—	42·7	27·9	6·2	48·0	1·0	0·004	0·059
											§35·9		§22·0			
	2·5	515	0·0242	350	—	—	—	—	—	44·1	14·3	5·9	46·3	1·0	0·042	0·031
											§20·4		§25·4			
Adipic	2·5	300	0·0041	150	2·0	7·1	0·04	5·2	2·4	4·0	3·8	2·0	8·3	2·2	—	—
	2·5	300	0·0142	144	0·5	6·8	0·2	3·1	2·2	15·0	7·3	5·4	8·6	1·8	—	—
	2·5	515	0·0222	350	0·2	2·7	0·2	4·1	2·1	32·2	17·0	4·0	24·2	0·6	—	—
	2·5	515	0·0222	350	0·1	5·2	0·4	11·4	—	30·9	14·6	4·4	24·4	0·7	—	—
	2·5	515	0·0222	350	—	—	—	—	—	22·2	13·7	4·0	21·8	0·4	—	—
Suberic	2·5	515	0·0186	350	0·2	1·2	0·1	1·2	2·1	5·3	6·2	4·8	7·1	1·4	—	—
	2·5	515	0·0186	350	—	—	—	—	—	7·8	9·5	4·2	10·3	1·9	—	—
	2·5	515	0·0275	350	—	—	—	—	—	9·8	8·1	7·6	17·7	1·6	0·010	0·008
											§8·8		§15·2			
	2·5	515	0·0242	350	—	—	—	—	—	8·7	7·1	3·7	22·1	1·5	0·015	0·020
Azelaic	2·5	515	0·0242	350	—	—	—	—	—	8·8	4·3	6·3	41·4	1·8	0·017	0·010
											§9·0		§24·3			
Laevulic	2·5	515	0·0142	350	—	—	—	—	—	14·3	17·9	2·9	34·8	1·2	—	—
	2	515	0·0058	350	—	—	—	—	—	15·7	11·9	2·2	29·1	0·8	—	—

* Percentages given are % carbon of original acid converted into the product concerned.
† Carbon dioxide evolved during the 1 hour's oxidation.
‡ Carbon dioxide evolved during subsequent steam-distillation after destruction of H_2O_2.
§ The lower figures give corrected values, allowance having been made for CO_2 retained as carbonate in the neutralised volatile acid distillates.

a cupric salt to the mixture; at the same time the proportion converted into carbonic and the lower fatty acids was much increased, the effect being most striking in the case of glutaric acid.

It will be seen from the figures given in Table I for adipic acid that a large increase in the amount of the hydrogen dioxide in the reaction mixture produced very little further oxidation in the absence of the copper salt. This agrees with the observation made by Dakin [1908] on the oxidation of the monobasic saturated fatty acids. Taking the percentage of the original acid converted into CO_2 and volatile acids as the criterion of the extent of oxidation, in the presence of the cupric salt, glutaric and adipic acids were more readily oxidised than succinic acid, both with a slight and with a large excess of the dioxide: in the latter case glutaric acid was almost completely converted into carbonic and the lower volatile fatty acids. A certain amount of carbonic acid was always given off during the steam-distillation of the product, markedly so in the case of glutaric and laevulic acids, probably owing to the decomposition of some unstable intermediate substance. Very little formic acid was present. Acetone in small amount was detected in the neutral volatile distillate after oxidation of glutaric acid; since acetone is hardly attacked by hydrogen dioxide in the presence of a copper salt, it is probable that little of the glutaric acid had undergone β-keto-oxidation.

When adipic acid was treated with a large excess of the dioxide in the presence of copper, no unchanged adipic acid was detected in the ether extract, but from 10 to 13 % of the total carbon had been converted into succinic and laevulic acids in the proportion of roughly 9 : 1. The succinic acid was identified by a mixed melting-point determination and the laevulic acid by the melting-point of its dinitrophenylhydrazone (200–202°), and a mixed melting-point with that of the same compound prepared from pure laevulic acid and melting at 202–204°. The formation of laevulic from adipic acid would be explained by oxidation at either the β- or γ-carbon atom, since in the dibasic adipic acid the β- and γ-derivatives are identical.

From the oxidation product of laevulic acid itself, carried out under similar conditions, small quantities of succinic acid and of acetone were obtained. Since on oxidation under these conditions, laevulic gave considerably less succinic acid than did adipic acid, laevulic acid was probably not the only intermediate substance formed. Succinic acid would also be formed by oxidation of the two α-carbon atoms, or possibly of the α- and δ-atoms, the α- and δ-positions in adipic acid being like the β- and γ-positions interchangeable. In the formation of succinic from glutaric acid, oxidation must have occurred at the α-atom (identical with the δ-position). The isolation of succinic acid from the higher dibasic acids in very much the same proportion is possibly due to a process of γ-oxidation by which a molecule of this acid is separated.

With a large excess of the dioxide, in the presence of a cupric salt at 60°, suberic and azelaic acids were less completely oxidised than the dibasic acids of lower molecular weight.

Under the above conditions from 20 to 40 % of suberic acid was converted into carbonic and the lower volatile fatty acids. The first two litres of the steam-distillate were neutralised and again distilled, the neutral distillate being collected as long as it gave a precipitate with dinitrophenylhydrazine hydrochloride solution. The first 200 ml. of this distillate were tested for acetaldehyde, and the subsequent distillate when treated with the reagent gave a hydrazone melting at 232–234°: this was very insoluble in most organic solvents but could be dissolved in glacial acetic acid and precipitated from this by water.

Microanalysis: (Found: C, 45·58; H, 3·72; N, 22·90 %. $C_{18}H_{18}N_8O_8$ requires C, 45·57; H, 3·80; N, 23·62 %). It was therefore identified as the di-dinitrophenylhydrazone of acetonylacetone: this substance was then prepared and the identity of the products established by determination of a mixed melting-point.

$\beta\beta$-Oxidation must therefore have taken place and acetonylacetone produced by elimination of CO_2 from the diketosuberic acid formed.

$$COOH.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.COOH \rightarrow COOH.CH_2.CO.CH_2.CH_2.CO.CH_2.COOH \rightarrow CH_3.CO.CH_2.CH_2.CO.CH_3$$

From the glacial acetic mother-liquors, a very small amount of a more soluble hydrazone, melting unsharply at 100°, was obtained, but the quantity was too small for further investigation. In the preparation of the dinitrophenylhydrazone from acetonylacetone, after the separation of the di-dinitrophenylhydrazone melting at 245°, a second hydrazone melting at 167° was separated from the glacial acetic mother-liquors.

The products from four experiments in each of which 5 g. of suberic acid had been oxidised were worked up together and from these a total of 12·06 g. of an ether-soluble non-volatile acid product were obtained. This was neutralised with *N* NaOH, the equivalent of the acid being 86.

No residue was obtained from the ethereal extract of the neutral solution. After acidification with hydrochloric acid, the bulk of the acids were removed by ether extraction, the aqueous solution evaporated to dryness and the residue extracted first with ether and then with absolute alcohol.

The material soluble in ether was washed three times with chloroform, leaving 4·06 g. of an insoluble crystalline acid, giving both on titration and on analysis of the silver salt an equivalent of 71. By fractional crystallisation from water of similar material, two substances were separated: (1) less soluble crystals identified by a mixed melting-point with suberic acid, and (2) a readily soluble substance melting at 178° which did not depress the melting-point of pure succinic acid when added to it. The 4·06 g. of material insoluble in chloroform, which had an equivalent of 71, probably therefore consisted of approximately 2·38 g. succinic and 1·68 g. unchanged suberic acid. About 10 % of the original suberic acid had remained unoxidised.

The chloroform solution contained 4·84 g. of a yellow oil, of which 2·35 g. were soluble in benzene: the oily residue from the benzene solution on standing in an evacuated desiccator deposited colourless crystals of which 0·13 g., M.P. 109–111°, remained after draining on a porous tile. The equivalent of these determined by titration was 84 and by analysis of the silver salt 93. Similar crystals were obtained in another experiment but with an equivalent of 102 (calculated from the decomposition of the silver salt).

Analysis: (Found: C, 50·63; H, 6·60 %. $C_8H_{14}O_5$ requires C, 50·52; H, 7·37 %. Equivalent, 95. $C_8H_{12}O_5$ requires C, 51·06; H, 6·33 %. Equivalent, 94).

The solution of the sodium salt gave no precipitate with dinitrophenylhydrazine hydrochloride and did not decolorise potassium permanganate solution; the melting-point of a specimen of α-hydroxysuberic acid (M.P. 110–112°) did not appear to be appreciably depressed by the addition of a small amount of the crystals now isolated, so that in spite of the low hydrogen percentage these crystals may have consisted of α-hydroxysuberic acid.

The tile which had been used for draining these crystals was extracted with ether, and an oil sparingly soluble in light petroleum was obtained. Its equivalent determined by titration in the cold was 116: by warming with excess of *N*/2

NaOH and titrating the excess with $N/2$ H_2SO_4 the equivalent was found to be 85. Decomposition of the moderately soluble silver salt gave an equivalent of 86. The high equivalent obtained by titration in the cold suggested the presence of a certain amount of the lactone of a hydroxydibasic acid.

	% C	% H	Equivalent
Microanalysis: Found	49·57	6·96	86
Hydroxysuberic acid ($C_8H_{14}O_5$)	50·52	7·37	95
Lactone of hydroxysuberic acid	55·82	6·98	172
Hydroxypimelic acid ($C_7H_{12}O_5$)	47·73	6·82	88
Lactone of hydroxypimelic acid	53·16	6·38	158

A mixture of 2/3 hydroxypimelic and 1/3 of the corresponding lactone contains 49·54 % C, 6·67 % H and would have an equivalent of 111, which would diminish to 88 on opening the lactone ring. These figures agree fairly well with those actually found but we were unable to find any evidence of pimelic acid in the reduction product. Possibly the low carbon percentage was due to the admixture of succinic acid or other impurity with hydroxysuberic acid.

0·227 g. of the oil was boiled for 3 hours with 5 ml. acetic anhydride, 2·5 g. hydriodic acid (sp. gr. 1·7) and 0·25 g. red phosphorus. The mixture was cooled and diluted: a tarry oil separated and the whole was evaporated nearly to dryness under reduced pressure. The black residue was dissolved in alcohol, excess of phosphorus filtered off and the dark brown filtrate boiled for 1½ hours with 6 g. zinc dust and 10 g. glacial acetic acid. The filtered solution was concentrated to about 10 ml. and then considerably diluted, causing the separation of an oil, which was extracted by ether and saponified by boiling with 40 ml. 5 % alcoholic potash for 1 hour. After diluting the product with water, ether extraction yielded about 0·06 g. of a white crystalline neutral substance melting at 57–58° when recrystallised from benzene and alcohol. This was slightly soluble in water the solution giving traces of a precipitate with a solution of dinitrophenylhydrazine hydrochloride and a slight silver mirror with ammoniacal silver nitrate.

Microanalysis: (Found: C, 76·57; H, 12·86 %. $C_{10}H_{20}O$ requires C, 76·93; H, 12·82 %. $C_9H_{18}O$ requires C, 76·05; H, 12·68 %). No further evidence as to its constitution was obtained.

After acidifying the alkaline aqueous solution, ether extraction yielded about 0·12 g. of a white crystalline acid which after recrystallisation melted at 106–108° and dissolved in water to the extent of less than 1 %, thus resembling in its properties the α-hydroxysuberic acid, crystals of which had originally been separated from this oily product: pimelic acid melts at 103° and is about 5 % soluble in water; the melting-point of suberic acid is 139–140° and its solubility less than 1 %.

On the whole the evidence points to hydroxylation of the suberic acid having taken place in the α- and γ-positions.

From the chloroform solution 2·04 g. of an oil insoluble in benzene were obtained which after standing for some time in an evacuated desiccator deposited 0·64 g. colourless crystals. These were separated on a porous tile and recrystallised from benzene: they melted at 130–131° and their equivalent determined by titration in the cold was 102, by analysis of the white silver salt, which was comparatively stable to light, 99. A solution of the sodium salt gave no hydrazone when treated with a solution of dinitrophenylhydrazine hydrochloride and did not decolorise a solution of potassium permanganate, so that the presence of a saturated hydroxy-acid was inferred.

Microanalysis of the silver salt: (Found: Ag, 52·11; C, 24·22; H, 2·70 %. $C_8H_{12}O_5Ag_2$ requires Ag, 53·46; C, 23·76; H, 2·97 %).

The percentage of silver was estimated from the weight of the residue in the boat after combustion. The melting-point was considerably higher than that of α-hydroxysuberic acid and since there was no evidence of lactone formation, the presence of γ-hydroxysuberic acid could be excluded. β-Hydroxysuberic acid, not hitherto described, may therefore have been present.

The oil from which these crystals had been separated contained 44·54 % C and 6·66 % H: the equivalent by titration was 98, by analysis of the silver salt 94: $C_7H_{12}O_6$ requires 43·75 % C; 6·25 % H; equivalent, 103. A dihydroxypimelic acid may therefore have formed the bulk of the oil.

On adding a solution of its sodium salt to a solution of dinitrophenylhydrazine hydrochloride, a very small amount of a precipitate, M.P. 85–90°, formed.

Microanalysis: (Found: C, 45·66; H, 4·01; N, 16·01 %. $C_{14}H_{16}O_8N_4$ (the dinitrophenylhydrazone of ketosuberic acid) requires C, 45·65; H, 4·35; N, 15·22 %); traces of a ketosuberic acid were therefore present in the oil.

The residue of the original oxidation product, which was insoluble in ether, was extracted with alcohol: most of this extract was soluble in water and consisted of a gummy substance which only very slowly reached constant weight and the nature of which was not determined.

The small amount of water-insoluble residue dissolved readily in alcohol and yielded a low-melting dark brown substance the constitution of which was not determined.

SUMMARY.

1. Laevulic acid and the dibasic acids, succinic, glutaric, adipic, suberic and azelaic, were readily oxidised at 60° by hydrogen dioxide in the presence of a cupric salt, a considerable proportion being broken down to carbonic and the lower volatile fatty acids.

2. Succinic acid was identified as a product of oxidation of glutaric, adipic, suberic and laevulic acids.

3. Azelaic and suberic acids were less completely broken down than the lower acids.

4. From glutaric acid, a small amount of acetone was isolated, showing that β-keto-oxidation had taken place.

5. From both glutaric and laevulic acids, considerable amounts of CO_2 were obtained during the steam-distillation of the oxidation product, pointing to the presence of an unstable intermediate product.

6. From adipic acid, laevulic acid was obtained in appreciable amount, showing that β-keto-oxidation had taken place.

7. Only in the case of suberic acid was definite evidence forthcoming of the presence of hydroxy-derivatives. The evidence available pointed to the presence of α- and γ-hydroxysuberic acids and possibly of the β-hydroxy-derivative. The presence of hydroxy-derivatives of pimelic acid as intermediate products could not be excluded. The identification of a very small amount of acetonylacetone showed that some ββ-keto-oxidation had taken place. The occurrence of a monoketosuberic acid and possibly of other keto-derivatives was also indicated.

REFERENCES.

Dakin (1908). *J. Biol. Chem.* **4**, 227.
Smedley-MacLean and Pearce (1934). *Biochem. J.* **28**, 486.
Verkade and Van der Lee (1934). *Biochem. J.* **28**, 31.

CXXVIII. STUDIES ON REVERSIBLE DEHYDROGENASE SYSTEMS.

I. THE REVERSIBILITY OF THE HYDROGENASE SYSTEM OF *BACT. COLI*[1].

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Stephenson and Stickland [1931] described an enzyme in various bacteria which could catalyse the reduction of oxygen, methylene blue, nitrate, sulphate or fumarate by molecular hydrogen. Consideration of this catalysis discloses a striking resemblance between hydrogenase and platinum black. Both the enzyme and the heavy metal catalyse the reaction

$$H_2 \rightleftharpoons 2H \rightleftharpoons 2H^{\cdot} + 2\epsilon.$$

In the presence of platinum black, this reaction proceeds reversibly (*cf.* the use of platinised platinum electrodes for the measurement of p_H). The experiments which follow show that the same equilibrium point between molecular and ionic hydrogen is reached in the presence of *Bact. coli* as in the presence of platinum black.

Experimental methods.

Bact. coli was grown in Roux bottles on a broth agar in the usual fashion. After two washings by centrifuging, the thick mass of bacteria was treated with saturated toluene water at room temperature for 15 minutes. This treatment sufficed to eliminate completely all endogenous reducing power.

The equilibrium between H_2 and $H^{\cdot}$ was measured with methylviologen (dimethyl-$\gamma\gamma'$-dipyridylium chloride), an indicator which lies in the range of the hydrogen electrode between p_H 7 and 9. The indicator was prepared according to the directions of Michaelis and Hill [1933], and the recrystallised chloride gave theoretical figures on elementary analysis.

The equilibrium potential was measured both colorimetrically and electrometrically. The colorimetric technique consisted essentially in setting up Thunberg tubes each containing buffer, bacterial suspension and methylviologen. The tubes were evacuated thoroughly and filled with oxygen-free hydrogen at a definite partial pressure according to the method of Stephenson and Stickland [1931]. Since the reaction proceeds very rapidly at 40°, the tubes were incubated at that temperature for an hour with frequent vigorous shaking. Then the temperature was lowered to 30° for the final measurements, at least 2 hours being allowed for equilibrium to be reached. For estimation of the degree of reduction a set of standards was prepared containing various fractions of the

[1] A preliminary note has already appeared in *Nature* (1934), **133**, 573.

[2] Beit Memorial Research Fellow.

total amount of indicator solution used in the experimental tubes. Both the total volume of fluid and the volume of bacterial suspension were the same in all the standard and experimental tubes. Reduction of the indicator in the standards was effected by adding a few crystals of sodium hydrosulphite and an excess of solid sodium carbonate.

The electrode vessels for the electrometric measurement of the equilibrium were constructed according to the design of Borsook and Schott [1931]. The only technical difficulty was the preparation of an agar bridge which could withstand a vacuum. By elimination of undissolved particles and air bubbles from the molten agar, bridges could be made to withstand a pressure of one atmosphere for the duration of several days, but the reliability rarely extended beyond the first few experiments.

The setting up of the experiment in the electrode vessel was identical with that described for the colorimetric experiments. Six electrode tubes were mounted on a special rack in an air-thermostat maintained at 30°. This rack permitted of vigorous shaking of the tubes. All the six tubes dipped into a large beaker of saturated potassium chloride which made contact with a reference saturated calomel electrode.

The potentiometry is described in detail in a paper by Green [1933]. The presence of the bacteria renders the potential recorded at the electrode somewhat unsteady, and readings of the potentiometer can at best be correct to about 1 millivolt.

The p_H of the buffer solutions used was measured by the quinhydrone and hydrogen electrodes. The buffers were made up to a concentration of $M/5$, and the final p_H of the bacterial suspension was assumed to be identical with that of the buffer alone.

Experimental results.

If *Bact. coli* and colloidal palladium are equivalent as catalysts, then for the same p_H and partial pressure of hydrogen the extent of reduction of methylviologen should be the same whichever catalyst is used. Table I summarises the results of this comparison. One set of Thunberg tubes contained 2 ml. of $M/5$ buffer, 0·5 ml. of $M/1000$ methylviologen and 1 ml. of bacterial suspension. A duplicate set was prepared in the same way except that the bacterial suspension had been previously heated to 100° for 15 minutes to inactivate the hydrogenase completely, and a drop of colloidal palladium suspension had been added. It is

Table I.

		Degree of reduction (%)	
p_H	Pressure of H_2 (mm. of Hg)	*Bact. coli* as catalyst	Palladium as catalyst
7·32	498	29	28
7·32	200	24	22
7·83	199	37	37
7·83	81	26	27
8·33	80	51	51
8·33	29	37	40
8·96	30	56	56
8·96	10·5	45	45

clear that the equilibrium-point is identical in the two sets of tubes. These measurements represent the average of comparisons carried out by two independent observers, and with the exception of one reading the two sets of values agreed to within 5 %.

If the potentials corresponding to the observed degree of reduction in Table I are calculated from the E_0 of the indicator, a uniform discrepancy of about 20 millivolts is found between the observed and theoretical potentials for the system $H_2 \rightleftharpoons 2H^{\cdot} + 2\epsilon$. This can only mean that the assumption of Michaelis and Hill [1933] that the E_0 of methylviologen ($-0{\cdot}446$ v.) is independent of p_H does not hold below p_H 9. Actually the measurements of these authors were confined to fairly alkaline p_H values, and their value for the neutral range is simply an extrapolation. One of us (D. E. G.) has noticed a similar discrepancy between the colorimetric and potentiometric values for the xanthine oxidase system in presence of methylviologen.

Table II contains a summary of the potentiometric measurements of the equilibrium $H_2 \rightleftharpoons 2H^{\cdot} + 2\epsilon$ in presence of *Bact. coli* and methylviologen, with

Table II.

p_H	Pressure of H_2 (mm. of Hg)	Potential (volts) Observed	Theoretical	Difference
7·02	600	−0·401	−0·408	+0·007
7·71	100	−0·433	−0·439	+0·006
7·71	306	−0·448	−0·453	+0·005
7·71	428	−0·455	−0·458	+0·003
8·12	49	−0·452	−0·455	+0·003
8·12	109	−0·463	−0·464	+0·001
8·12	161	−0·470	−0·470	0·000
8·12	237	−0·477	−0·475	−0·002
8·12	598	−0·489	−0·487	−0·002
8·23	105	−0·464	−0·470	+0·006
8·23	138	−0·475	−0·473	−0·002
9·10	20	−0·484	−0·487	+0·003

variation of the hydrogen and hydrogen ion concentrations. The remarkable agreement between the observed values of the potential and those calculated from the hydrogen electrode equation

$$E_h = -\frac{RT}{F} p_H - \frac{RT}{2F} \log P_{H_2},$$

gives adequate proof that hydrogenase functions as a perfect catalyst, *i.e.* it increases the rate of attainment of the equilibrium but does not alter the equilibrium-point.

SUMMARY.

Bact. coli has been shown to catalyse the reaction

$$H_2 \rightleftharpoons 2H^{\cdot} + 2\epsilon$$

in a completely reversible way. The hydrogenase system is the most negative reversible oxidation-reduction system as yet described in living cells.

REFERENCES.

Borsook and Schott (1931). *J. Biol. Chem.* **92**, 535.
Green (1933). *Biochem. J.* **27**, 1044.
Michaelis and Hill (1933). *J. Gen. Physiol.* **16**, 859.
Stephenson and Stickland (1931). *Biochem. J.* **25**, 205.

CXXIX. THE ACTION OF INHIBITORS ON THE CATECHOL OXIDASE OF POTATOES.

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Attempts have been made to classify the phenolases into groups such as the *catechol oxidases*, *tyrosinases*, and *laccases* [Raper, 1928], but the classification is made difficult by the lack of satisfactory methods of characterising the enzymes [Onslow and Robinson, 1928].

The present work was undertaken with the object of testing whether the catechol oxidases of different origin behave similarly towards inhibitors, or whether they show any marked differences of behaviour which might be used as a further guide in classifying these enzymes.

Catechol oxidases are found widely distributed in plants, fungi and insects. In the animal kingdom, a phenolase specific for 3 : 4-dihydroxyphenylalanine ("Dopa oxidase") and a phenolase of horse's heart muscle [Wieland and Lawson, 1931] have been investigated. Other systems effecting the oxidation of adrenaline and phenolases present in melaniferous tumours have been described. Summaries of the literature are given by Raper [1928], Onslow and Robinson [1928], Hizume [1924] and Oppenheimer and Kuhn [1931].

Resorcinol was chosen as a suitable inhibitor, since preliminary experiments with the catechol oxidase of potatoes showed that the inhibition with resorcinol appeared to be of a simple competitive type, as it could be reduced by increasing the substrate concentration.

Experimental.

Cathechol oxidase of potatoes. The catechol oxidase of potatoes (called tyrosinase by Raper [1928]) was prepared by the method of Szent-Györgyi [Keilin, 1928] and was further purified by adsorption on aluminium phosphate at p_H 6 and elution with phosphate buffer at p_H 8. The aluminium phosphate [Tsuchihashi, 1923] was added to the enzyme solution until most of the enzyme was adsorbed. (This was shown by centrifuging off a small sample of the clear solution and testing for catechol oxidase by shaking in a test-tube with a few drops of 2 % catechol.) After adsorption, the aluminium phosphate was centrifuged off and washed twice with distilled water, and the enzyme was eluted by adding disodium hydrogen phosphate solution until the mixture was at p_H 8. About 50 % of the enzyme was lost in the process of adsorption, but the resulting preparation, which was nearly colourless, appeared to be very pure, and could be kept for several weeks in the ice-chest without any considerable loss of activity.

This preparation brought about a very rapid oxidation of catechol and its derivatives, but the system was found to be unsuitable for making kinetic measurements as the reaction came to a standstill after the first few minutes owing to the inhibition of the system by one of the reaction products (Fig. 1).

This auto-inhibition was probably due to the *o*-quinone formed in the reaction

$$C_6H_4(OH)_2 + O_2 \rightarrow C_6H_4O_2 + H_2O_2$$

since it was greatly reduced by adding to the system substances such as aniline or *o*-phenylenediamine which readily combine with *o*-quinones. Other substances found to be effective in decreasing the auto-inhibition were *m*- and *p*-phenylenediamine, glycine and alanine (Fig. 1).

For the purpose of making kinetic measurements with inhibitors *M*/20 aniline was therefore added to the system, which in the presence of *M*/125 catechol, and *M*/50 phosphate buffer p_H 6·8, gave a regular and reproducible reaction velocity. The anilinoquinone formed during the reaction separated out as a red precipitate.

Enzyme strength. As an arbitrary unit of the enzyme strength the amount of enzyme was chosen which in the presence of *M*/125 catechol, *M*/50 phosphate buffer p_H 6·8, and *M*/20 aniline (total volume 5 ml.) gave an oxygen uptake of 100 μl. in 10 minutes when shaken in air in a Barcroft apparatus at 20°. This enzyme unit is referred to as Catechol Unit or C.U. throughout the text.

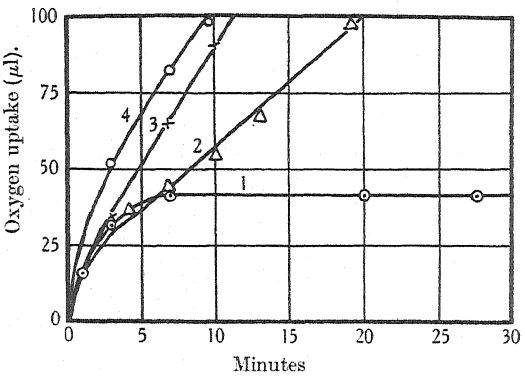

Fig. 1.

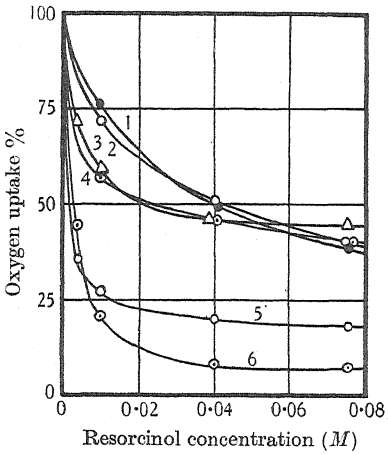

Fig. 2.

Fig. 1. In each experiment *M*/125 catechol, *M*/150 phosphate buffer p_H 6·8, and 0·9 C.U. potato catechol oxidase. Total volume 5 ml. Temperature 20°. 1. No addition. 2. *M*/10 glycine. 3. *M*/20 aniline. 4. 0·4 % *o*-phenylenediamine.

Fig. 2. In each experiment *M*/125 catechol, *M*/50 phosphate buffer p_H 6·8, *M*/20 aniline. Total volume 5 ml. Temperature 20°. 1. *Polyporus hispidus*. 2. *Tenebrio molitor*. 3. Potato. 4. *Agaricus campestris*. 5. *Sambucus nigra*. 6. *Syringa vulgaris*.

Inhibition by resorcinol.

The enzyme preparations used for comparison were prepared by the alcohol extraction method of Onslow and Robinson [1928] from fresh specimens of *Syringa vulgaris* (lilac), *Sambucus nigra* (elder), *Agaricus campestris* (mushroom), and *Polyporus hispidus*. The preparation from *Tenebrio molitor* (mealworm) was purified by repeated precipitation with alcohol and dialysis.

The enzymes were all used at approximately the same strength (0·3 C.U./ml.) under the conditions described for determining the enzyme strength of the

potato catechol oxidase, and the oxygen uptake in the presence of a series of concentrations of resorcinol was measured. The oxygen absorbed between the third and the thirteenth minutes was taken for comparison. The oxygen uptake plotted against the inhibitor concentration gave the curves shown in Fig. 2.

The inhibition curves obtained in this way appeared to depend very little on the degree of purity of the enzyme, since the curves obtained with crude potato juice agreed closely with the curves for the highly purified enzyme.

It can be seen at once from the curves (Fig. 2) that the catechol oxidases from different sources are not identical, but show marked specific differences in their behaviour. It is noticeable that the curves for *Syringa vulgaris* and *Sambucus nigra*, neither of which contains tyrosinase, lie together. While the usual methods of characterising enzymes, such as the measurement of p_H optimum or thermolability, must depend largely on the nature of the accompanying colloids, the characteristic selective inhibition of the phenolases by an inhibitor such as resorcinol probably depends more on the specific nature of the active centres of the enzymes.

Other inhibitors.

Apart from the inhibition by H_2S and HCN described by Keilin [1928] the catechol oxidase of potatoes is comparatively resistant towards narcotics and the usual enzyme poisons. The action of a number of substances was investigated by measuring the oxygen uptake of the potato enzyme at p_H 8 and 20° in their presence.

Of these substances phloroglucinol, *p*-nitrophenol, α-naphthol, dimethylaniline, thiolacetic acid, cysteine, salicylaldehyde and benzyl alcohol acted as inhibitors, while the following substances had little or no effect: urethane, methylurethane, methyl, ethyl, amyl and octyl alcohols, glycerol, glucose, pyridine, piperidine, diethylamine, urea, *o*-cresol, salicylic acid, *p*-quinone, quinhydrone, guaiacol, phenolphthalein, formaldehyde, acetaldehyde, phenylglyoxal, diacetyl, vanillin, sodium cholate, sodium *n*-hexanoate, sodium *n*-octanoate, fluorescein, haematin, *p*-rosaniline, methylene blue, picric acid, sodium pyrophosphate, uric acid, amygdalin, benzonitrile and quinine sulphate. The substances tested were brought to p_H 8 by addition of dilute sulphuric acid or sodium hydroxide before being tested.

It was observed that the addition of glycine or aniline to the system reduced the inhibition by benzyl alcohol, resorcinol and phloroglucinol, but this phenomenon was not further investigated.

Secondary oxidation of o*-phenylenediamine.*

It has been shown qualitatively that the *o*-quinones formed by the oxidation of catechol derivatives can effect the secondary oxidation of gum guaiacum, the "Nadi Reagent" and certain amino-acids [Szent-Györgyi, 1925].

o-Phenylenediamine was found to be oxidised in a similar manner, and some experiments were carried out to show the concentration of catechol derivative required for the reaction to take place.

M/125 *o*-phenylenediamine (freshly recrystallised) underwent no measurable oxidation on shaking for 8 hours with the potato catechol oxidase at p_H 6·8. On the addition of 0·02 mg./ml. of adrenaline or dihydroxyphenylalanine to

the system, an easily measurable absorption of oxygen took place, and the solution darkened in colour owing to the oxidation of the *o*-phenylenediamine (Fig. 3).

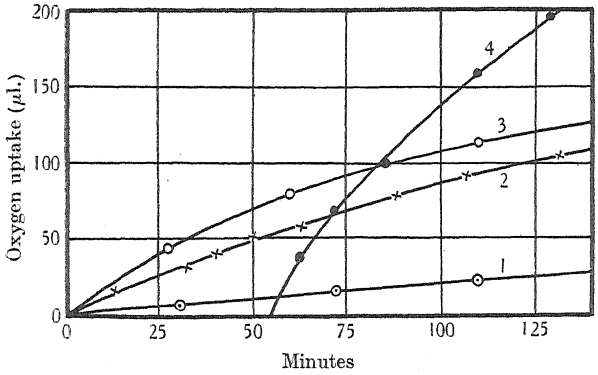

Fig. 3. In each experiment $M/125$ *o*-phenylenediamine, $M/50$ phosphate buffer p_H 6·8, and 0·54 C.U. potato catechol oxidase. Total volume 5 ml. Temperature 20°. 1. 0·02 mg./ml. adrenaline. 2. 0·1 mg./ml. adrenaline. 3. 0·06 mg./ml. dihydroxyphenylalanine. 4. 0·2 mg./ml. dihydroxyphenylalanine (dihydroxyphenylalanine in 4 added after 54 minutes).

Secondary oxidation of thiolacetic acid.

Since —SH compounds are known to be present in tissues which contain the catechol oxidase, it seemed probable that this system would be able to bring about the secondary oxidation of —SH compounds to the disulphides.

Attempts to demonstrate this oxidation by adding a solution of thiolacetic acid were unsuccessful, since the catechol oxidase was almost completely inhibited by —SH compounds. The —SH concentration was therefore reduced by adding to the system an aldehyde such as formaldehyde, with which —SH compounds form dissociable complexes [Pirie and Pinhey, 1929]:

$$R.CHO + R'.SH \rightleftharpoons R.CH(OH).SR'$$

It was found that the addition of formaldehyde greatly reduced the inhibition of the catechol oxidase and allowed a rapid oxidation of the thiolacetic acid to take place. The following figures give the results of four experiments at 20°. Total volume 5 ml. in each experiment.

Enzyme, 14·6 C.U./ml. (ml.)	—	0·3	0·3	0·3
$M/10$ phosphate buffer, p_H 6·0 (ml.)	1	1	1	1
$M/50$ Na thiolacetate (ml.)	1·5	1·5	1·5	1·5
30 % formaldehyde (ml.)	0·3	0·3	—	0·3
$M/5$ catechol (ml.)	0·2	—	0·2	0·2
Time of experiment (mins.)	240	164	270	120
Oxygen absorbed, μl.	0	0	94	343
Thiolacetate ($N/50$ iodine)	1·5	1·5	1·0	0·1

The catechol and formaldehyde present interfered with the direct iodimetric estimation of the thiolacetic acid at the end of the experiment. The procedure adopted was to precipitate the thiolacetic acid with lead acetate at p_H 5, centrifuge off and wash the precipitate with water. On treating with potassium iodide and sulphuric acid, the precipitate was decomposed. The lead iodide was centrifuged off and washed, and the thiolacetic acid in solution was titrated directly with $N/50$ iodine.

Oxidation of monohydric phenols.

In addition to effecting the oxidation of catechol derivatives, preparations of the catechol oxidase of potatoes also bring about the oxidation of monohydric phenols, such as *p*-cresol, *m*-cresol, phenol and tyrosine. The reaction is specific, since phenols such as guaiacol, *o*-cresol, salicylic acid, thymol, α-naphthol and in general those phenols which on oxidation are likely to give quinol derivatives rather than catechol derivatives, are not attacked [Onslow and Robinson, 1925; 1926; 1928; Pugh and Raper, 1927].

Raper [1928] and Pugh [1930] hold the view that one enzyme only is involved in the oxidation both of catechol derivatives and of monohydric phenols.

Onslow and Robinson [1928] observed that the rate of oxidation of monohydric phenols was greatly increased by the addition of a catechol derivative. They concluded that the oxidation of the monohydric phenols is a secondary oxidation, effected by a reaction product of the catechol oxidase system. In her earlier papers Onslow concluded that the intermediate substance which oxidises the monohydric phenol is an organic peroxide, but later she held that it might be either hydrogen peroxide or an *o*-quinone. She assumed that a second enzyme might catalyse the secondary oxidation of the monohydric phenols.

Pugh [1929] has shown that the addition of a trace of a catechol derivative or of an *o*-quinone also accelerates the rate of oxidation of monohydric phenols with the phenolase preparation from mealworms, but she does not accept Onslow and Robinson's explanation as to the mechanism. She has also shown that the oxidation of monohydric phenols is accelerated by the addition of a trace of hydrogen peroxide, but this might be due to the formation of *o*-quinone by the partial oxidation of the monohydric phenol by the hydrogen peroxide, catalysed by peroxidase present in the enzyme preparation.

Onslow and Robinson [1928] claimed that they had effected a partial separation of the system into two enzymes, but this was disputed by Pugh [1930].

Acceleration by catechol derivatives. In the present work Onslow and Robinson's colorimetric experiments on the effect of catechol derivatives on the oxidation of monohydric phenols have been confirmed and extended by quantitative measurements of the oxygen uptake, using the phenolase system of the potato. *p*-Cresol was used as a substrate in preference to tyrosine, in which the initial oxidation is followed by a series of reactions connected with the amino-acid grouping, which introduces an unnecessary complication.

The oxidation of *p*-cresol in the presence of crude potato juice was very rapid and showed a regular oxygen uptake. With the purified enzyme, on the other hand, the rate of oxidation was initially very slow, but increased autocatalytically to a high value (Fig. 4).

The initiation of the reaction was strongly accelerated by the addition of boiled potato juice, or of a trace of a catechol derivative (potato juice contains catechol derivatives) such as adrenaline, dihydroxyphenylalanine or catechol. The acceleration produced by even 0·4 mg./litre adrenaline could be measured.

As a possible explanation of the action of catechol derivatives Onslow and Robinson suggested that the hydrogen peroxide formed by their oxidation might cause the secondary oxidation of the monohydric phenols:

$$\text{Catechol derivative} + O_2 + (\text{catechol oxidase}) \rightarrow o\text{-quinone} + H_2O_2 \qquad \text{I.}$$

$$H_2O_2 + \text{monohydric phenol} + (\text{peroxidase}) \rightarrow \text{catechol derivative} + H_2O \qquad \text{II.}$$

On this view the oxidation of the monohydric phenol by hydrogen peroxide would be catalysed by peroxidase present in the enzyme preparation and would

give rise to a catechol derivative which would in turn be oxidised by the catechol oxidase. The process would be continuous and might be regarded as a type of chain reaction.

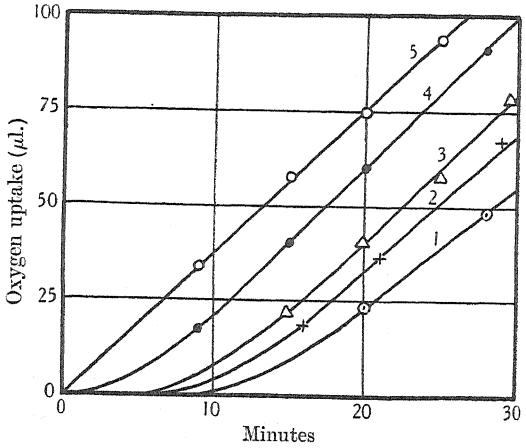

Fig. 4. In each experiment $M/125$ *p*-cresol, $M/50$ phosphate buffer p_H 6·8, and 2·4 C.U. potato enzyme. Total volume 5 ml. Temperature 20°. 1. No addition. 2. $M/250$ glycine. 3. 0·4 mg./litre adrenaline. 4. 0·5 ml. boiled potato juice. 5. 8 mg./litre adrenaline.

Against this view it was observed that the specimens of potato catechol oxidase purified by adsorption were almost free from peroxidase. On testing for peroxidase with gum guaiacum and hydrogen peroxide the enzyme solution gave only a faint blue coloration after standing for 10 minutes.

It was further observed that the addition of 0·3 % sodium pyrophosphate or saturation with pyrrole, which very strongly inhibit vegetable peroxidase [Elliott, 1932], had no appreciable effect on the rate of oxidation of *p*-cresol. It must be concluded that in the oxidation of monohydric phenols the peroxidase system is not primarily concerned.

The autocatalytic nature of the curve with the purified enzyme could be explained if during the purification a trace of inhibitor were accidentally introduced, and the reaction velocity slowly increased as the inhibitor was oxidised by the *o*-quinone produced during the reaction. This possibility was rendered improbable by the fact that the enzyme had the same properties when further purified by different methods, *e.g.* by adsorption, dialysis or precipitation with alcohol. Further, it was observed that when the enzyme was "activated" by the addition of a trace of catechol, it was found to be no longer activated if allowed to stand until the *o*-quinone formed had decomposed.

Inhibition of the oxidation of monohydric phenols. The most probable explanation of the action of catechol derivatives appeared to be Onslow and Robinson's suggestion that the monohydric phenols are oxidised by the *o*-quinones formed:

Catechol derivative + O_2 + (catechol oxidase) → *o*-quinone + H_2O_2 I.

Monohydric phenol + *o*-quinone + (dehydrase) → 2 mols. catechol derivative II.

Here again the reaction would be continuous, since reaction II regenerates the catechol derivative required for reaction I.

Attempts to test this view by showing that a trace of *o*-quinone could be added in the place of the catechol derivative were inconclusive, since *o*-quinones are very unstable in aqueous solution, and after standing only for a few minutes

the solution contained an appreciable amount of catechol derivatives [Willstätter and Müller, 1911]. The addition of sodium β-naphthoquinonesulphonate, an *o*-quinone derivative which is relatively stable in aqueous solution, produced only a slight acceleration.

This view of the action of *o*-quinones was therefore tested by adding to the system substances which combine with *o*-quinones. It was found that the oxidation of *p*-cresol or tyrosine was completely inhibited by 0·5 % *o*-phenylenediamine, *M*/1000 sodium bisulphite, 1 % potassium iodide or *M*/10 aniline for a period of over 5 hours, although the catechol oxidase was still active, since a rapid oxygen uptake occurred on adding catechol.

This inhibition by substances that combine with *o*-quinones gives additional evidence that *o*-quinones play an essential part in the oxidation of monohydric phenols. The view that one enzyme catalyses the direct oxidation of both catechol derivatives and monohydric phenols by molecular oxygen [Raper, 1928; Pugh, 1930] does not explain the extreme sensitivity of the latter, but not the former, reaction to traces of *o*-quinones and to substances which combine with *o*-quinones.

The instability of the *o*-quinones in solution makes it difficult to obtain direct experimental proof, but the oxidation potentials of the *o*-quinones are sufficiently high for the oxidation of the monohydric phenols, and the available evidence is consistent with the view that this oxidation takes place.

Summary.

1. Catechol oxidase preparations from *Syringa vulgaris*, *Sambucus nigra*, *Agaricus campestris*, *Polyporus hispidus*, *Tenebrio molitor* and potato were compared by testing the extent to which they were inhibited by varying concentrations of resorcinol. Preparations of different origin showed marked specific differences in their behaviour. An improved method was obtained for preparing stable preparations of the catechol oxidase of potatoes.

2. Conditions were found under which the catechol oxidase system of potatoes effected the secondary oxidation of *o*-phenylenediamine and of thiolacetic acid.

3. Measurements of the rate of oxidation of *p*-cresol by the catechol oxidase of potatoes showed the reaction to be strongly accelerated by substances which produce *o*-quinones, and strongly inhibited by substances which combine with *o*-quinones. The reaction would therefore appear to be essentially a secondary oxidation in which the formation of an *o*-quinone is involved, rather than a direct oxidation of *p*-cresol by the enzyme.

The author wishes to thank Prof. Sir Frederick Gowland Hopkins and also Mrs Adair for their interest and advice.

References.

Elliott (1932). *Biochem. J.* **26**, 1281.
Hizume (1924). *Biochem. Z.* **147**, 216.
Keilin (1928). *Proc. Roy. Soc. Lond.* B **104**, 206.
Onslow and Robinson (1925). *Biochem. J.* **19**, 420.
—— —— (1926). *Biochem. J.* **20**, 1138.
—— —— (1928). *Biochem. J.* **22**, 1327.

Oppenheimer and Kuhn (1931). Die Fermente. (Thieme, Leipzig.)
Pirie and Pinhey (1929). *J. Biol. Chem.* **84**, 321.
Pugh (1929). *Biochem. J.* **23**, 456.
—— (1930). *Biochem. J.* **24**, 1442.
—— and Raper (1927). *Biochem. J.* **21**, 1370.
Raper (1928). *Physiol. Rev.* **8**, 245.
Szent-Györgyi (1925). *Biochem. Z.* **162**, 399.
Tsuchihashi (1923). *Biochem. Z.* **140**, 63.
Wieland and Lawson (1931). *Liebig's Ann.* **485**, 193.
Willstätter and Müller (1911). *Ber. deutsch. chem. Ges.* **44**, 2171.

CXXX. THE ACTION OF CRYSTALLINE VITAMIN B_1 ON THE RESPIRATION OF POLYNEURITIC TISSUES *IN VITRO*.

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THE connection of vitamin B_1 with tissue oxidation has been considered for many years, and recent work has strongly suggested that this vitamin is intimately associated with the oxidation of carbohydrates. Gavrilescu and Peters [1931] showed that the oxygen uptake of minced polyneuritic pigeon's brain tissue in the presence of added glucose was significantly lower than that of the normal tissue. More recently [Gavrilescu *et al.*, 1932] this has been shown to be the case in the presence of added lactate as well, an average diminution of 33 % being found for cerebrum, and 45 % for the optic lobes; in the presence of succinate however there is no difference between the levels of oxidation of normal and avitaminous tissues, while the residual uptake, *i.e.* without added substrate, also shows no significant difference. The addition of small amounts of crystalline vitamin B_1 restores the oxygen uptake of the avitaminous tissue in the presence of lactate almost to the normal level. It is concluded that this *in vitro* effect of the vitamin on the respiration of brain tissue is specifically connected with the glucose-lactate enzyme systems, and this action is now referred to as the catatorulin effect of the vitamin [Passmore *et al.* 1933].

It seems to be well established therefore that there exists a specific defect in the oxidative mechanism of the brain in B_1 avitaminosis, but with regard to the other tissues the position is not at present clear. The literature concerning the level of respiration in normal and avitaminous tissues other than brain is confused and contradictory, as may be seen from Westenbrink's review of the subject [1932, 1]. In the fifteen investigations reviewed the only positive results, as Westenbrink has pointed out, which survive statistical examination are those of Ahlgren [1923] on minced pigeon muscle and of Tsukamoto [1928] on pigeon liver slices[2].

Galvao and Cardoso [1932] investigated, by the methylene blue method, the reducing powers of normal and avitaminous tissues and of tissues from animals in a state of inanition. They found that the reducing power of muscle tissue from animals suffering from inanition was lower than that of the normal tissue, but that there was no lowering of the reducing power of muscles from polyneuritic animals when excessive loss of weight was prevented by forced feeding on polished rice; in the case of liver they found no difference even between normal animals and animals in a state of inanition.

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[2] For further discussion of the experiments of Abderhalden and Vlassopoulos [1931] see Gavrilescu *et al.* [1932].

Westenbrink, impressed with the fact that Tsukamoto's positive results had been obtained with liver slices in contrast with the minced tissues which had been used by other workers, has recently [1932, 2] repeated the experiments of Tsukamoto, but has been unable to find any decrease in the oxygen uptake of liver slices either from pigeons or rats.

It is perhaps worthy of mention that in none of the experiments reviewed by Westenbrink, except those of Drummond and Marrian [1926] on the reducing powers of the tissues for methylene blue, nor in Westenbrink's own experiments, was any added substrate used, the tissue being placed merely in Ringer solution or in a Ringer buffered by phosphate or bicarbonate.

In view of the conflicting results obtained in the comparison of the tissue respiration of normal and avitaminous animals, the work of Peters and his colleagues on the catatorulin effect of vitamin B_1 in polyneuritic pigeon's brain tissue seems to provide a more decisive method for determining the presence of such an oxidative lesion in other avitaminous tissues. By analogy with brain, the addition of vitamin B_1 to suspensions of these tissues respiring in lactate should result in an increased oxygen uptake, if such an oxidative lesion exists. Such a procedure avoids the controversial question of the level of tissue respiration in vitamin B_1 deficiency, and also has the advantage that it is less dependent on the general state of nutrition of the animal. The following experiments therefore were primarily undertaken not in the hope of settling whether there was a specific lowering of cell-respiration in polyneuritic tissues, but to determine whether added vitamin B_1 showed any catatorulin activity in the tissues of polyneuritic pigeons other than the brain.

Method.

The method employed has been to kill the birds by guillotining. The organ to be investigated is then quickly dissected out and removed from the body; any surrounding connective tissue is carefully dissected away and the organ gently wiped free from blood with filter-paper. In dissecting out the kidney it has been found most convenient to pluck the back of the abdomen and then to remove the entire piece of the bony posterior abdominal wall in front of which the kidney lies; by this means the kidney can be removed with the least amount of bleeding. Muscle, heart, liver and kidney have been investigated, the muscle employed being the pectoralis major.

In all cases except where subsequently mentioned the tissues have been finely chopped up with scissors: as far as was possible to judge the tissues were minced to the same extent each time. In those experiments in which sections were used the tissue was sliced free-hand with a razor moistened with the tissue fluid only, as recommended by Harrison [1933].

The oxygen uptake was measured in bottles of the Barcroft-Dixon type containing 3 ml. of Ringer-phosphate solution at p_H 7·3 (made up according to the usual formula used in this laboratory) to which neutralised sodium lactate, prepared from recrystallised zinc lactate, had been previously added in the amount required to make the final concentration of lactate 0·033 *M*. To the bottles containing vitamin 2·8 ml. of Ringer-phosphate-lactate solution were added before the addition of the tissue; the subsequent addition of a further 0·2 ml. of the medium containing 1–2 γ of the crystalline vitamin [Kinnersley *et al.* 1933] bringing the final volume up to 3 ml. The bottles were weighed before and after the addition of the tissue; after the second weighing the tissue

was finely broken up with a flat-ended glass rod and the vitamin added to those bottles where required. Rolls of filter-paper moistened with 2*N* KOH were used to absorb the CO_2. The bottles were then twice evacuated to 70 mm. and filled with oxygen. The temperature of the bath was 38°.

All the avitaminous birds, which were fed according to the usual procedure adopted in this laboratory, showed well-marked symptoms of head-retraction at the time of use. The amounts of tissue employed in each case varied with the different tissues on account of differences in their rates of oxygen consumption: the approximate average amounts per bottle were 70 mg. for kidney, 100 mg. for muscle and liver, and 130 mg. for heart. All the values given are the averages of duplicate estimations, the agreement being in nearly every case within 7 %.

Results.

It was found that with avitaminous kidney the addition of the vitamin produced in all cases a large and striking increase in the oxygen uptake measured over four successive half-hour periods, but with muscle, heart and liver the effect when present was always small and not actually statistically significant.

The results obtained with avitaminous muscle, heart and liver, with and without the addition of vitamin are shown in Table I.

The oxygen uptakes are expressed in microlitres of oxygen per g. of tissue per hour over four successive half-hour periods: this method of expression has been used in order to render the results more comparable with those of other workers on this problem; for those who prefer the modern nomenclature $2000\,\mu$l./g./hour $= Q_{O_2}$ 10 approximately. It can be seen from this table that the oxygen uptake of these organs is little affected by the addition of the vitamin: in the muscle and heart experiments the bottles containing vitamin had in some cases a lower uptake than the controls, and in those cases where the vitamin did bring about an increase, this increase was always small, reckoned either as the excess amount of oxygen taken up in μl. per g. of tissue per hour or as the percentage rise over the control, and was not statistically significant[1]. In the case of the liver the addition of the vitamin produced in every case a small increase in the oxygen uptake, but here also though the actual amount of oxygen taken up was greater than in the case of muscle or heart, the percentage increase was of the same order and was again not statistically significant except possibly for the fourth half-hour period. Since the vitamin effect, when present, was in every case so slight, and in view of the fact that statistical examination of the results shows that the increase is definitely not significant, it was thought unnecessary to do more than five of these experiments on each tissue. Four additional experiments were done using slices of liver, but no effect was obtained by this method.

Table II shows the results obtained with avitaminous kidney with and without the addition of vitamin. As in the previous experiments the amount of vitamin added was 1–2 γ per bottle. It may be seen that with kidney the addition of the vitamin produced in every case a large increase in the oxygen uptake, obviously of quite a different order from the effects obtained with the other tissues, amounting in the fourth half-hour period to 885 μl., or 58 % of the oxygen uptake without added vitamin.

[1] The significance of the results obtained in this research was determined by means of Fisher's "t" test.

Table I. *Avitaminous heart, muscle and liver.*

O_2 uptake (μl. O_2/g./hour).

Heart.

Exp.	Periods (hours) 0–½	½–1	1–1½	1½–2
8. L.	850	430	345	255
L.+V.	835	460	412	265
11. L.	725	447	277	182
L.+V.	772	464	274	189
12. L.	837	475	252	193
L.+V.	872	506	351	202
14. L.	715	341	178	165
L.+V.	804	379	213	158
37. L.	705	335	179	156
L.+V.	695	405	214	167
Average L.	765	405	245	190
L.+V.	795	445	295	195
Average increase	+30	+40	+50	+5

Muscle.

5. L.	2555	794	257	—
L.+V.	2410	937	305	—
35. L.	750	415	294	232
L.+V.	770	460	262	179
37. L.	780	300	146	76
L.+V.	700	310	140	116
38. L.	1415	480	213	118
L.+V.	1535	510	222	138
39. L.	545	193	114	95
L.+V.	515	202	154	105
Average L.	1210	435	205	130
L.+V.	1190	485	215	135
Average increase	−20	+50	+10	+5

Liver.

30. L.	1105	800	597	442
L.+V.	1185	868	667	506
34. L.	1265	890	650	—
L.+V.	1290	890	660	—
35. L.	2200	1330	905	675
L.+V.	2210	1340	1020	760
37. L.	1465	1090	715	585
L.+V.	1765	1205	830	708
38. L.	1870	1230	900	600
L.+V.	1960	1280	980	715
Average L.	1580	1070	755	575
L.+V.	1680	1120	830	670
Average increase	+100	+50	+75	+95

Table II. *Avitaminous kidney.*

O_2 uptake (μl./g./hour).

Exp.	Periods (hours) 0–½	½–1	1–1½	1½–2
11. L.	2120	2060	1845	1485
L.+V.	2335	2500	2385	2220
12. L.	2100	2075	1760	1525
L.+V.	2450	2480	2405	2340
14. L.	2355	2275	1885	1555
L.+V.	2735	2935	2735	2660
19. L.	2265	2120	1790	1585
L.+V.	2820	2780	2790	2630
24. L.	1870	1875	1725	1475
L.+V.	2340	2140	2490	2215
34. L.	2365	2130	1840	1575
L.+V.	2630	2650	2515	2450
Average L.	2180	2090	1810	1535
L.+V.	2550	2580	2550	2420
Increase (μl. O_2)	370	490	740	885
% increase	17	23	41	58

It is also noteworthy that whereas the respiration of the avitaminous kidney *in vitro* falls off steadily, the addition of the vitamin maintains the respiration at an almost constant level. In view of this catatorulin effect of the vitamin on the kidney as well as on the brain it is of interest to compare the rates of respiration of normal and avitaminous kidney tissue in the presence of lactate. The results obtained with normal kidney without added vitamin are shown in Table III.

Table III. *Normal kidney.*

O_2 uptake (μl./g./hour).

Exp.	Periods (hours) 0–½	½–1	1–1½	1½–2
13	3120	3455	3085	3225
17	2440	2510	2425	2485
18	2830	2865	2680	2860
27	2470	2460	2775	2265
28	3075	3145	2965	3150
43	3570	3345	3475	3265
Average	2920	2965	2900	2875

It will be seen that the respiration of normal kidney, like that of the avitaminous kidney in the presence of added vitamin, maintains itself at a fairly constant level (Fig. 1). Statistical examination of these figures also shows that the level of oxygen uptake of the avitaminous kidney is significantly lower than that of the normal, while Table III shows that the lowered level of respiration of the avitaminous kidney is appreciably restored by the addition of the vitamin. Four experiments were done to determine whether added vitamin had any effect on the respiration of normal kidney in the presence of lactate; it was found that there was no effect.

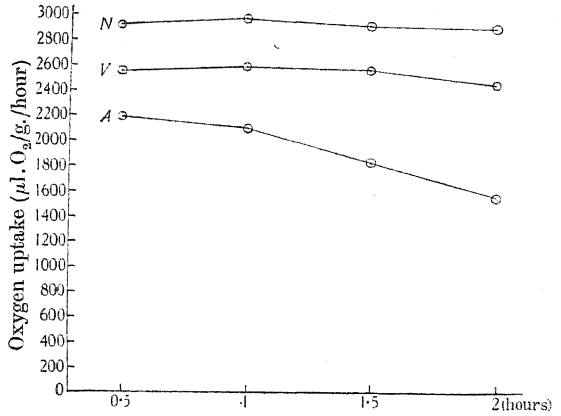

Fig. 1. Mean rate of oxygen uptake of minced kidney in presence of lactate.
N = normal; A = avitaminous; V = avitaminous + vitamin.

Discussion.

It seems justifiable to conclude from the results of this research that the addition of vitamin B_1 to muscle, heart or liver tissue from polyneuritic pigeons has no significant effect on the oxygen uptake *in vitro*, but that it shows a marked catatorulin effect on polyneuritic kidney respiring in the presence of lactate.

As already pointed out the primary object of this research was to investigate, not the rate of respiration of normal and avitaminous tissues, but the catatorulin effect of the vitamin in polyneuritic tissues other than brain. But in view of the discovery of this striking effect on polyneuritic kidney, it seemed essential to determine whether the extra oxygen uptake produced by the added vitamin was a restoration of a lowered level of respiration to the normal level. The results show that, as in the case of brain, the respiration of the polyneuritic kidney *in vitro* in the presence of added lactate is markedly lower than that of the normal tissue, the lowering becoming more pronounced in the third and fourth half-hour periods. There exists therefore in the kidneys of polyneuritic pigeons in opisthotonus some oxidative lesion which is capable of being largely repaired *in vitro* by the addition of vitamin B_1. No experiments have yet been done to decide the stage in avitaminosis at which the kidney lesion commences.

It is possible that this kidney lesion may be connected in some way with the oedema which is sometimes present in polyneuritic birds, but it is certain that other factors must also be concerned, since no obvious oedema was present in any of the birds used during this research.

Summary.

1. The respiration of minced muscle, heart, liver and kidney tissues from polyneuritic pigeons has been studied in the presence of lactate with and without the addition of small amounts of vitamin B_1.

2. The addition of the vitamin to polyneuritic muscle, heart or liver has no significant effect on the oxygen uptake of these tissues *in vitro*, but in the case of polyneuritic kidney the addition of the vitamin brings about a large and statistically significant increase in respiration.

3. The respiration of the polyneuritic kidney in the presence of lactate is significantly lower than that of normal kidney; the addition of the vitamin largely restores this defect.

I wish to express my sincere gratitude to Prof. Peters for his constant interest and advice during the course of this work. I am also indebted to Mr H. W. Kinnersley for supplying the vitamin concentrates used, and to Mr R. B. Fisher for assistance with the statistical examination of the results.

REFERENCES.

Abderhalden and Vlassopoulos (1931). *Pflüger's Arch.* **226**, 808.
Ahlgren (1923). *Skand. Arch.* **44**, 186.
Drummond and Marrian (1926). *Biochem. J.* **20**, 1229.
Galvao and Cardoso (1932). *Pflüger's Arch.* **229**, 422.
Gavrilescu, Meiklejohn, Passmore and Peters (1932). *Proc. Roy. Soc. Lond.* B **110**, 431.
—— and Peters (1931). *Biochem. J.* **25**, 1397.
Harrison (1933). *Biochem. J.* **27**, 1501.
Kinnersley, O'Brien and Peters (1933). *Biochem. J.* **27**, 232.
Passmore, Peters and Sinclair (1933). *Biochem. J.* **27**, 842.
Tsukamoto (1928). *Tôhoku J. Exp. Med.* **11**, 142.
Westenbrink (1932, 1). *Arch. Néerl. Phys.* **17**, 239.
—— (1932, 2). *Arch. Néerl. Phys.* **17**, 549.

CXXXI. PYRUVIC ACID AS AN INTERMEDIARY METABOLITE IN THE BRAIN TISSUE OF AVITAMINOUS AND NORMAL PIGEONS.

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The recent discovery of a pyruvate reaction in lactate solutions in which avitaminous pigeon's brain has respired *in vitro* for 2 hours [Peters and Sinclair, 1933] is of interest in relation to the action of vitamin B_1, because addition of this reduces the amount of the abnormal substance (for references see Gavrilescu *et al.* [1932] and Meiklejohn *et al.* [1932]). More generally, identification of pyruvate in these circumstances lends support from an unexpected source to the recent conceptions of this substance as a normal intermediary in the metabolism of carbohydrate in the animal (see especially Embden *et al.* [1933] and Meyerhof and Kiessling [1933]). Otherwise these views depend entirely upon work with added sulphite or fluoride. There is also involved the even more fundamental problem of synthesis of carbohydrate from lactic acid, since the indication is that vitamin B_1 is related to the oxidative synthesis of lactate. With this idea in mind, we have devoted attention in the main to phenomena occurring in lactate solutions, as we are more likely so to approximate to conditions favouring the synthesis. The experiments described in this paper have been made with minced brain and in phosphate solutions to facilitate manipulations and to obtain better duplicate control. The conditions are frankly artificial and narrowed for the study of the one point, but it is most unlikely that qualitative errors have been imposed by our conditions. The presence of the substance reacting like pyruvate is equally induced by sections of avitaminous brain and was found by Sinclair (personal communication) to occur with bicarbonate solutions.

We have proved that the nitroprusside-reacting substance is actually pyruvic acid and have obtained quantitative data relating extra pyruvate disappearance in presence of vitamin to extra oxygen uptake to find out whether pyruvate disappears as a direct or indirect effect of the vitamin[1].

Experimental.

Pyruvic acid is known to bind bisulphite. It can be readily shown that bisulphite-binding substances (B.B.S.) (*a*) do not appear during the respiration of normal brain tissue (*b*) appear during that of polyneuritic tissue, and (*c*) disappear with addition of vitamin. Bisulphite-binding substances however do appear during the respiration of normal brain tissue in the presence of iodoacetate.

[1] A preliminary account has appeared in *J. Physiol.* **81**, 22 P.

Unless otherwise stated the minced[1] brain tissue from normal or polyneuritic pigeons (with opisthotonos symptoms) has been shaken in phosphate Ringer-lactate or pyruvate solution at p_H 7·3 [see Passmore *et al.*, 1933] in differential manometers filled with oxygen: in all cases anhydrous sodium pyrophosphate (brought to p_H 7·3 with HCl) was added in a concentration of 0·0094 *M* per bottle [Peters and Sinclair, 1933]. At the end of the respiration period (usually 2 hours) at 38° to each cooled bottle was added 0·9 ml. of 25 % trichloroacetic acid. After standing 1 hour the contents were filtered and the bottles washed out with 2 ml. of 5 % trichloroacetic acid.

Table I.

Bisulphite-binding substances (B.B.S.).

Results are expressed in ml. *N*/100 I per g. tissue
(1 ml. = 0·44 mg. pyruvic acid).

I. Residual.

Exp.		Initial	R.P. 2 hours	R.P. + V. 2 hours	Difference produced by vitamin
533	Normal	—	1·18	—	—
562	Avitaminous	—	1·64	1·60	0·04
663	,,	—	2·08	1·69	0·39
535	,,	1·17	—	—	—
564	,,	—	2·80	1·74	1·06
565	,,	—	1·98	1·74	0·24
		Average	**2·12**	**1·69**	**0·43**

II. Lactate.

Exp.		Initial	L. 1 hour	L. 2 hours	L. + Iodo.	L. + Fl.
545	Normal	—	—	2·75	—	2·72
531	,,	0·92	2·32	(3·30) (3·34)	—	—
533	,,	—	—	2·87	—	—
534	,,	—	—	3·10	—	—
541	,,	—	—	3·69	7·25 (0·0018 *M*)	3·04
592	,,	—	—	4·23	8·2 (0·0005 *M*)	3·8
			Average	**3·33**	**7·72**	**3·19**

(Iodoacetic acid concentration in brackets. Fluoride 1 mg./ml. NaF (0·024 *M*).

Avitaminous.

Exp.	L. 1 hour	L. + V. 1 hour	Difference	L. 2 hours	L. + V. 2 hours	Difference	L. + Fl. 2 hours
535	6·88	5·5	1·38	10·2	7·14	3·06	5·8
539	—	—	—	9·37	5·63	3·74	—
537	5·96	3·78	2·18	7·16	3·93	3·23	—
551	6·6	5·4	1·20	8·95	6·58	2·37	—
553	—	—	—	8·85	6·41	2·44	—
554	—	—	—	9·30	5·23	4·07	—
557	—	—	—	9·88	6·03	3·85	—
558	—	—	—	10·0	5·10	4·90	—
560	—	—	—	11·10	5·33	5·77	—
569	—	—	—	7·45	4·69	2·76	—
580	—	—	—	9·8	5·28	4·52	5·33
Average	**6·48**	**4·89**	**1·59**	**9·28**	**5·58**	**3·70**	**5·56**

[1] In unpublished results we have found that a large quantitative difference may be induced in respiration by variations in temperature of mincing. The data as to temperature have not been included in this paper, because no qualitative effect seems to have been produced upon the quotient studied.

The combined filtrates were treated with N NaOH to bring to p_H 2·0 and made up with water to a final volume of 15 ml. for each bottle containing originally 3 ml. of Ringer *plus* brain tissue. 5 ml. of this solution were then treated with 0·5 ml. of M $NaHSO_3$ and allowed to stand for 15 min. [Clift and Cook, 1932]. Excess bisulphite was removed with $N/10$ and then $N/100$ iodine; 5 ml. of saturated $NaHCO_3$ were added and the liberated bisulphite was titrated with $N/100$ iodine. The results are given in Tables I and I A.

Table I A.

Abstract of results (averages).

	Initial	R.P. 2 hours	R.P. + V. 2 hours	Diff.	L. 1 hour	L. + V. 1 hour	Diff.
Residual.							
Normal (1)	—	1·18	—	—	—	—	—
Avitaminous (5)	1·17	2·12	1·69	0·43	—	—	—
Lactate.							
Normal (6)	0·92	—	—	—	2·32	—	—
Avitaminous (11)	—	—	—	—	6·48	4·89	1·59

	L. 2 hours	L. + V. 2 hours	Diff.	L. + Fl. 2 hours	L. + Fl. + V. 2 hours	Diff.	L. + Iodo.	L. + Gl.	L. + Gl. + Iodo.
Lactate.									
Normal (6)	3·33	—	—	3·19	—	—	7·72	3·18	5·67
Avitaminous (11)	9·28	5·58	3·70	5·56	3·90	1·66	—	—	—

Results in Table I are the mean of duplicate estimations; they agree within ±2 % for L. (avit.), (±0·01 ml. iodine for the equivalent of 1·0 ml. bottle contents). For smaller amounts of pyruvate the error is proportionately greater. V. = in all cases, 2γ crystalline vitamin B_1 hydrochloride per Barcroft bottle [Kinnersley *et al.*, 1933].

Substances combining with bisulphite are present in very small amounts in normal brain and increase slightly during respiration in lactate (not more than 0·4 mg. per g.). The amounts formed with avitaminous brain are of a different order (ten times as much); they decrease 39 % with added vitamin.

A comparison of experiments in Ringer-phosphate only, with those in lactate show a slight increase of B.B.S. with lactate in normal, but a large increase for avitaminous brains. Hence lactate is essential for the formation of the B.B.S. which are decreased in amount by added vitamin. Before proceeding further, it is necessary to know how much of the B.B.S. is really pyruvic acid. The method of Clift and Cook [1932] of heating in alkali to remove substances other than pyruvic acid did not prove satisfactory in our hands, and could not be so certain as isolation of the 2:4-dinitrophenylhydrazone [Case, 1932].

Case's method involves the following steps:

1. Addition of 2:4-dinitrophenylhydrazine to the trichloroacetic acid solution.
2. Extraction of mixed hydrazine and hydrazones with ethyl acetate.
3. Neutralisation with $CaCO_3$.
4. Evaporation and extraction with toluene.
5. Extraction with Na_2CO_3.
6. Acidification and extraction with ethyl acetate.
7. Evaporation to dryness.
8. Estimation in solution in alcoholic potash.

This has been modified as follows. Stage 1. Same; 5 ml. 1 % hydrazine in 2N HCl added to 20·0 ml. trichloroacetic acid solution.

2. Same; at least 3 extractions, and often more.

3. Successive extractions with alkaline phosphate, 1/3 vol. The first extraction with saturated alkaline phosphate (Na), and subsequent with $M/15$.

4. Acidification with HCl and extraction with ethyl acetate.

5. Repeat stage 3.

6. Repeat stage 4, making acid to p_H 2·5 approx.

7. As Case.

8. As Case.

We found that our extracts did not give good results by Case's method unmodified. Since the pure hydrazone of pyruvic acid was found to be completely extracted by alkaline phosphate we proceeded as in stages 3 and 4, continuing the extraction with alkaline phosphate until fresh extracts were colourless, or showed only a faint brown-yellow colour, due to traces of hydrazine. With this exception other stages were repeated until colourless. The trace of hydrazine carried over into 4 is removed almost completely in 5 and 6. The combined ethyl acetate solutions from 2–3 bottles (equivalent to 200–300 mg. tissue) are conveniently made up to 5–6 ml. 1–2 ml. (Ostwald pipette) can be evaporated in a small measuring flask to 0·1 ml., cooled and made up to the mark with the alcoholic potash. Standards containing 0·5 mg. per 1·0 ml. ethyl acetate have been used for comparison ($\equiv$0·15 mg. pyruvic acid). The 0·1 ml. ethyl acetate left assists solution in the alcoholic potash and minimises the risk of hydrolysing the hydrazone. It appears to have no influence upon the determination provided that the estimation is performed reasonably soon.

The method has the great advantage that heating is always avoided up to stage 6, and if necessary after this by evaporation *in vacuo*. Hence unstable hydrazones such as that of oxaloacetic acid [Clift and Cook, 1932] should be detected more easily. Stages 2–6 for 4 estimations take approximately 4 hours.

Stage 7 reaches a high accuracy with 0·5 mg. or even less of hydrazone. Stages 2–6 give a good recovery of pyruvic acid 2:4-dinitrophenylhydrazone added at stage 2 to the combined ethyl acetate extracts. Examples are given in Table II.

Table II. *Recoveries.*

1. Pyruvic acid 2:4-dinitrophenylhydrazone added at stage 2. Final ethyl acetate phase made up to 6·0 ml. approx. and 2·0 ml. taken for analysis in all except *c* (3·0 ml.). 1·0 mg. $\equiv$ 0·328 mg. pyruvic acid.

	Added mg.	Found mg.	Corr. mg.
(*a*)	1·0	1·02	1·0
(*b*)	1·0	1·02	1·0
(*c*)	1·0	1·03	1·0
(*d*)	1·5	1·49	1·48

2. Pyruvic acid added at stage 1.

	Added mg.	Found mg.	Corr. mg.
(*e*)	0·492	0·494	0·488
(*f*)	0·492	0·496	0·490
(*g*)	0·984	0·984	0·981
(*h*)	0·984	0·960	0·957

As stated previously a trace of hydrazine accompanies the hydrazone, making a difference of approx. 0·01 mg. hydrazone (0·003 mg. pyruvic acid) per 1·0 ml. of final phase taken. This can be allowed for as a blank, if desired. Our slight modification is presumably more specific in its extraction than the Na_2CO_3 stage, but we have not investigated this.

Table III gives the results of estimations by the modified Case 2:4-dinitrophenylhydrazone method upon solutions from some of the experiments given in Table I. The experiments are given in order and probably increase in accuracy;

Table III. *Substrate lactate. Pyruvic acid, estimated by 2:4-dinitrophenylhydrazone method. mg./g.*

Exp.	L.	L.+V.	L. − (L.+V.)	L. %*	L.+V. %*	Diff. O_2 uptake	µl./mg. P.A.	Remarks
554	3·32	1·66	1·66	81	72	1345	815	M.P. hydrazone low, 193°
557	2·90	1·68	1·22	67	63	962	790	M.P. 216°
558	3·67	1·57	2·10	84	69	1010	480	M.P. low, 204°
560	3·30	1·37	1·93	68	58	1115	580	(No pyrophosphate)
569	3·31	1·79	1·52	102	88	1162	765	
580	3·88	1·89	1·99	90	82	1020	515	
Average	**3·40**	**1·66**	**1·73**	**82**	**72**	**1102**	**657**	

* % of B.B.S. estimated as pyruvic acid 2:4-dinitrophenylhydrazone.

Exp. 590. Samples of avitaminous cerebrum in bottles as follows: 4 contained L. only, 4 L. + V. All were treated similarly, but two of each were removed from the bath after the preliminary shaking period, cooled and treated with trichloroacetic acid. The remainder were shaken 1 hour.

	min.	Pyruvic acid: As B.B.S.	Pyruvic acid: As 2:4-dinitro.	% as pyruvic acid	Weight tissue	− P.A./g.	Diff. P.A.	O_2/g.
L.	12	0·185	0·075	41	0·174	0·43	—	—
L.	60	0·502	0·398	78	0·205	1·91	1·48	1710
L.+V.	12	0·157	0·068	50	0·215	0·32	—	—
L.+V.	60	0·290	0·218	72	0·194	1·08	0·76	2070

Diff. P.A. = 1·48 − 0·76 = 0·72 mg.
Diff. O_2 = 360 µl.
O_2/P.A. = 500.

Note. Single estimations only were performed in these experiments owing to the small amount of pyruvic acid present.

in all cases L. and L.+V. have been extracted side by side. The last experiment (590) is given separately; an attempt was made to estimate the influence of the initial period upon the pyruvic acid disappearance, when there is no estimation of oxygen. The ratio $+O_2/-$pyruvic acid is within the limit for the others. Pyruvic acid evidently forms a large proportion of the B.B.S. and disappears in presence of added vitamin. Some 20 % of B.B.S. are not pyruvic acid, and this proportion increases as the result of the vitamin action. From this it may be inferred that one of the substances into which pyruvic acid is transformed is a non-acidic substance containing an aldehyde or ketone group.

In Exps. 551, 557, after one or two further purifications by repetition of stages 5 and 6, pure pyruvic acid hydrazone separated upon evaporation of the ethyl acetate phase, M.P. 216°, which showed no alteration in a mixed M.P. Traces of other hydrazones which give a red colour with alcoholic potash often accompany the pyruvic acid hydrazone, giving this a red tint and a lower M.P.; the amount of such substances is small, so that there seems no doubt that the main substance concerned is pyruvic acid.

Pyruvate as substrate.

The conclusion that the vitamin catalyses disappearance of pyruvate as well as increased O_2 uptake can be confirmed by substitution of pyruvate for lactate in the respiring system. This is in striking contrast to lactate, extra disappearance of which was not shown to take place in presence of vitamin [Meiklejohn, 1933].

In the experiments recorded in Table IV (A, B) the amounts of pyruvate present in the Ringer-phosphate solution were 5·0 mg. approx. (0·019 *M*).

Table IV. (A) *Pyruvate, titrated as B.B.S.*

(Data for sum of duplicate bottles.)

	P.					P. + V.			
Exp.	Initial pyruvate mg.	Weight tissue g.	Pyruvate 2 hours mg.	Pyruvate (mg.) disappearing per g.	O_2 uptake per g. 2 hours μl.	Weight tissue g.	Pyruvate 2 hours mg.	Pyruvate mg./g.	O_2 uptake per g. 2 hours μl.
562	10·1	0·2302	8·9	5·2	1220	0·2192	8·1	9·15	2450
563	[9·91]	0·220	8·9	[4·6]	1270	0·1944	8·7	[6·25]	2290
564	10·86	0·2370	9·55	5·52	1350	0·1787	9·6	7·06	2050
565	[10·36]	0·1983	9·45	[4·6]	2180	0·1589	9·2	[7·3]	3165
567	11·5	0·1966	9·95	7·9	1700	0·2112	9·4	9·95	2540
582	10·3	0·1975	9·8	2·53	1635	0·1850	9·45	4·6	2855
588	11·95	0·1664	11·55	2·4	2170	0·1697	10·83	6·6	3595
590	11·95	0·1971	11·17	3·95	1785	0·2049	10·3	8·05	2870
567*	11·5	0·2025	10·2	6·42	1275	0·2233	9·2	10·3	2850

* With lactate present also.

(B) *Pyruvate, estimated by hydrazone method*.*

	P.		P. + V.	
Exp.	Pyruvate 2 hours mg.	% B.B.S.	Pyruvate 2 hours mg.	% B.B.S.
563	8·4	94·5	8·15	94
564	9·2	96	8·4†	87·5†
565	8·65	91·5	8·25	90
567	9·37	94	9·3	99
567‡	9·75	95·5	8·9	97
582	9·08	92·5	8·56	90·5
588	10·7	92·5	10·11	93·5

* Mean of duplicate observations agreeing within 2 %, in exps. 567, 582, 588. Remainder single observations.

† Some known to have been lost. ‡ With lactate present also.

Table IV (B) shows that there is much more satisfactory agreement between the B.B.S. by titration and the hydrazone estimations than for lactate. A summary of the essential differences is given in Table V.

Table V. *Substrate pyruvate. The relation between pyruvate disappearing and O_2 uptake due to added vitamin B_1 (differences only).*

Exp.	Pyruvate mg./g.	O_2 uptake ml./g.	O_2/P.A.
562	3·72	1230	330
563	1·55	1020	658
564	1·45	700	482
565	2·53	985	389
567	1·93	840	435*
582	1·95	1220	625
588	3·95	1425	361
590	3·95	1085	275
Average	2·63	1063	443

* With lactate also present 380.

A point needing discussion arises in connection with some of the data in Table III. If the amounts of tissue are approximately equal the initial amount of pyruvate need not be known; if not, error may be introduced. Expressed mathematically:

If c = initial concentration of pyruvate,

a_1, a_2 = amounts present after time t, in pyruvate and pyruvate-vitamin respectively,

b_1, b_2 = weights of tissue (mg.),

k = constant,

d = difference wanted, all being in suitable units,

we have made the general assumption that

$$(c - a_1) = kb_1, \text{ etc.} \qquad \text{.....(1),}$$

$(c - a_1)\frac{100}{b_1}$ and $(c - a_2)\frac{100}{b_2}$ = disappearance of pyruvic acid per 100 mg. of tissue for P. and P. + V. respectively.

Hence
$$d = \frac{100}{b_1 . b_2}[c\,(b_2 - b_1) + a_2 b_1 - a_1 b_2] \qquad \text{.....(2).}$$

Unless $b_1 = b_2$, it is necessary to know c.

In most experiments c is known; in Exps. 563, 566, its value was doubtful owing to changes which pyruvic acid appears to undergo at slightly alkaline reaction. Clift and Cook [1932] heat in alkaline solution to remove bisulphite-binding compounds other than pyruvic acid, and we have found that over-neutralisation with NaOH has little effect upon the pyruvate provided that the solution is dilute. But direct addition of N NaOH to pyruvic acid, a procedure adopted in some experiments to minimise volume, may be attended by 50 % changes in bisulphite-binding power. This was not realised at first.

In Exps. 563 and 565, where low values were obtained for the titration of bisulphite-binding substances, we have substituted for c by use of the equation

$$(c - a_1) = k_1 b_1 \qquad \text{.....(3).}$$

Other known errors in estimating the final quotient lie in the initial 12 minute period in which pyruvic acid disappears and the oxygen uptake is not estimated. In general this will not be serious, as it affects both P. and P. + V., and the oxygen uptakes differ less at first than afterwards.

The extra amounts of pyruvic acid disappearing are of the same order though larger than those with lactate, though the extra oxygen uptakes are very similar in the two sets of experiments, *i.e.* the catatorulin effect is not changed by the substrate. Addition of lactate to pyruvate also caused no change in behaviour in Exp. 567. The consistent vitamin effect with pyrophosphate present is in striking contrast to the earlier work of Meiklejohn *et al.* [1932], who found in absence of pyrophosphate an abnormally lowered respiration with pyruvate and a most variable vitamin effect. From this it was concluded that pyruvate was not a stage in the oxidation of lactic acid, and that another factor was needed to secure pyruvate oxidation. That the failure to interact was not due to blockage by excess lactate in the avitaminous brains was proved by the use of iodoacetic acid. The lowered respiration has been a constant feature of these experiments, but the consistency of the catatorulin effects indicates that pyrophosphate takes the place of the missing factor.

Pyruvic acid is the first organic substance which has been proved to disappear *in vitro* as the result of action of vitamin B_1, this change accompanying the extra oxygen uptake. Much of the evidence presented up to this point is consistent with the idea that the vitamin acts directly upon the pyruvate. We hesitate to take this at its face value. In spite of the constancy of oxygen uptake, there is not much agreement between the O_2/P.A. values. Though the errors involved in the estimation of small differences admittedly reach a high value in Table V, the variations appear to be outside the limits of error. It must be remembered that in the earlier experiments, the vitamin effect failed with pyruvate in tissue which reacted in presence of lactate. Further we have found in a few experiments at p_H 6·6 that pyruvate functions in place of pyro-

phosphate in inducing good oxygen uptake with vitamin, but the disappearance of pyruvate and hence the ratio is again variable (Table VI). (*Cf.* Peters and Sinclair [1933] for behaviour at p_H 6·6.)

Table VI. *Respiration in pyruvic acid at* p_H *6·6 (estimated as B.B.S.).*

	P.					P. + V.			
Exp.	Initial* pyruvate mg.	Weight* tissue g.	Pyruvate* (2 hours) mg.	Pyruvate disappearing mg. per g.	O_2 uptake μl. per g.	Weight* tissue g.	Pyruvate* (2 hours) mg.	Pyruvate disappearing mg. per g.	O_2 uptake μl. per g.
563 (*b*)	[9·81]	0·2454	8·7	[4·6]	1060	0·1991	8·5	[6·5]	1820
570 (*a*)	11·95	0·2194	10·5	6·6	1410	0·1949	9·9	10·5	2425
572 (*a*)	11·5	0·2633	10·4	4·2	1270	0·2519	9·7	7·15	1800
(*b*)	11·5	0·1785	10·4	6·15	1215	0·1972	10·1	7·1	1715
575 (*a*)†	[5·1]	0·1866	4·35	[4·6]	1220	0·1866	4·15	[5·0]	1505
(*b*)†	—	0·1858	4·15	[4·6]	1165	0·2001	3·96	[5·8]	1600

Summary.

Exp.	Pyruvate mg./g.	O_2 μl./g.	O_2/P.A.
563 (*b*)	−1·9	+ 760	400
570 (*a*)	−3·9	+1015	260
572 (*a*)	−3·0	+ 530	180
(*b*)	−1·0	+ 500	500
575 (*a*)†	−0·4	+ 285	700
(*b*)†	−1·2	+ 435	360

* Expressed per sum of duplicate bottles.
† Lactate also present.
(*a*) Without pyrophosphate.
(*b*) With pyrophosphate.

The balance of the evidence inclines to the view that pyruvate disappearance is really consequent upon some other change, and that the vitamin catalyses some other reaction. The sum total of these reactions indicates that about two molecules O_2 are taken up for one molecule of pyruvic acid disappearing; the average ratio for the lactate experiments is 660 μl./mg., for pyruvate 450 μl./mg.; calculated for quotient 2 mol. O_2/1 mol. P.A. = 506.

According to Sinclair [1933] the R.Q. for the extra oxygen uptake due to vitamin in lactate-phosphate solution is 1·6. Hence the average for lactate (660 = 2·5 mol. O_2 approx.) should give 3·75 mol. CO_2 per 1 mol. pyruvic acid disappearing.

For complete oxidation we should have

$$CH_3.CO.COOH + 5O = 3CO_2 + 2H_2O,$$

and R.Q. = 1·2. There is therefore evidence that more CO_2 is being evolved than is required for complete combustion of the pyruvic acid, which indicates entry of some other substance into the oxidation system.

We have the curious paradox that at p_H 7·3 pyrophosphate seems to be needed for pyruvate extra respiration, whereas at p_H 6·6 pyrophosphate is required for lactate extra respiration; at this p_H however pyruvate will function in place of pyrophosphate in restoring catatorulin effect in the presence of lactate. In this complex effect, there appears to be no co-operation of α-glycerophosphate [Peters and Sinclair, 1933], which might have been expected upon the Meyerhof scheme. It is possible that both pyruvate and pyrophosphate are acting as H acceptors at p_H 6·6.

The substance formed from pyruvate in the avitaminous brain is not yet known. Since Meiklejohn [1933] showed that there was no change in lactate accompanying the extra O_2 uptake, and since there is some variation in the ratio O_2/P.A., it seems to be unlikely that lactate is the main substance. In any case, such a conversion involves a reduction.

The simplest explanation of the accumulation of pyruvic acid in the avitaminous tissue and its disappearance in presence of vitamin B_1 is that pyruvic acid is a normal intermediary stage in some phase of carbohydrate metabolism. This is in essence the Embden-Meyerhof view. The phenomena embodied in the new anaerobic fermentation schemes evidently take some part in aerobic respiration too; according to Meyerhof and Kiessling [1933] fluoride should interfere with the appearance of pyruvic acid from phosphoglycerate, and iodoacetate with its disappearance. Consistently with this, we find that no increased pyruvate appears in normal brain tissue respiring in lactate, though a large increase occurs in the presence of iodoacetate (see Exps. 541 and 592, Tables I and VII). Fluoride (Exps. 535, 580, Table I) much reduces the pyruvate formed

Table VII.

Normal brain.

Respiration (μl./g.)	Substrate	(Hours) 0–½	½–1	1–1½	1½–2	Total (2 hours)
	L.	2660	2360	1900	1430	4170
	L. + iodo.	2460	1580	870	340	2625
	L. + fluoride	1470	1310	715	520	2005

Pyruvate estimations.

Substrate	B.B.S.	2:4-dinitro.	%	Tissue weight	− P.A./g.	O_2
L.	0·315	0·255	81	0·170	1·5	4170
L. + iodoacetate	0·665	0·512	77	0·185	2·77	2625
L. + fluoride	0·132	—	—	0·0792	1·33	2005

L. − L. + iodoacetate. Ratio O_2/P.A. = 825.

by avitaminous brain in lactate solutions. Both poisons reduce the O_2 uptake. Other known origins for pyruvate can probably be dismissed with exception of oxaloacetic acid [Krebs, 1933]. Origin from alanine seems mainly confined to kidney [Krebs, 1932], and that from lactic acid [Banga *et al.* 1932; Barron and Hastings, 1933] is not blocked by fluoride (personal communication from Dr E. Boyland). The appearance of pyruvate during respiration of brain tissue *in vitro* and its inhibition by fluoride provide evidence of the re-entry of lactate into some fermentation system; consideration of this must be postponed until we have more precise data of the action of these poisons upon the catatorulin effect.

Note. The figures throughout are given in terms of tissue wet weight, and not as Q_{O_2}, because there seems to be no advantage in the use of this symbol in this comparative work. The values can be readily translated by considering the dry weight to be 20 % of the wet weight values. $Q_{O_2} = 10 \equiv 2000$ ml./g.

Summary.

1. Disappearance of pyruvic acid and of bisulphite-binding substances accompanies the extra oxygen uptake induced by the action of crystalline vitamin B_1 in pigeon's brain tissue respiring *in vitro*. Estimations have been made by the method of Clift and Cook and by a modified Case method.

2. Pyruvate also disappears when substituted for lactate.

3. The average value for the quotient extra O_2 uptake/pyruvic acid disappearing approximates to 506, representing 2 mols. O_2 taken up to 1 mol. pyruvic acid disappearing, but there are wide variations, which lead to the belief that disappearance of pyruvate is an indirect effect of vitamin action.

4. The phenomena are consistent with the view that pyruvic acid is a normal intermediary in the metabolism of pigeon's brain tissue. In agreement with the Embden-Meyerhof fermentation scheme, it accumulates with respiring normal brain tissue in presence of iodoacetate and not of fluoride.

We are indebted to Mr H. W. Kinnersley for a supply of the vitamin solutions, to R. W. Wakelin for skilful help and to the Medical Research Council for grants in aid of the research.

REFERENCES.

Banga, Szent-Györgyi and Vargha (1932). *Z. physiol. Chem.* **210**, 228.
Barron and Hastings (1933). *J. Biol. Chem.* **100**, 155.
Case (1932). *Biochem. J.* **26**, 753.
Clift and Cook (1932). *Biochem. J.* **26**, 1788.
Embden, Deuticke and Kraft (1933). *Klin. Woch.* **12**, 343.
Gavrilescu, Meiklejohn, Passmore and Peters (1932). *Proc. Roy. Soc. Lond.* B **110**, 431.
Kinnersley, O'Brien and Peters (1933). *Biochem. J.* **27**, 232.
Krebs (1932). *Klin. Woch.* **42**, 1744.
—— (1933). *Z. physiol. Chem.* **218**, 157.
Meiklejohn (1933). *Biochem. J.* **27**, 1310.
—— Passmore and Peters (1932). *Biochem. J.* **26**, 1872.
Meyerhof and Kiessling (1933). *Biochem. Z.* **264**, 40.
Passmore, Peters and Sinclair (1933). *Biochem. J.* **27**, 842.
Peters and Sinclair (1933). *Biochem. J.* **27**, 1910.
Sinclair (1933). *Biochem. J.* **27**, 1927.

Note added in proof by R. A. P. In a recent paper [Birch and Mann (1934), *Biochem. J.* **28**, 622] it is stated that we assumed that the oxidation affected was that of lactic acid. Reference to the papers in question, particularly Gavrilescu *et al.* [1932, p. 445], will convince that we were pedantically careful to write oxygen uptake in presence of lactate. In regard to the later criticism of the conclusions drawn from the previous pyruvate experiments, these authors seem to have overlooked the important point that some of the experiments then performed showed only trifling increases with pyruvate and vitamin as compared with pyruvate alone. Later in their paper, they refer to our original statement that 1/10 pigeon dose produced a maximum increase. Unfortunately this has not been corrected in print, though a correction can be inferred from reference to Passmore *et al.* [1933], where about 1/2 pigeon dose produced a nearly maximum effect. The crude preparation originally used was found upon subsequent test to contain more vitamin than had been supposed.

The melting-points in the above paper are corrected. There is some doubt in the literature as to the exact melting point of pyruvic acid 2:4-dinitrohydrazone. The most usual figure obtained is 216°. We have, however, had in our hands a specimen which melted at 218°. It is hoped that sufficient of the compound from the lactate experiments may become available for further confirmation by analysis.

CXXXII. VITAMIN B_3.

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(*Received May 2nd, 1934.*)

Of the components of the vitamin B complex, three have been considered necessary for the nutrition of the pigeon: vitamins B_1, B_3 and B_5. Williams and Waterman [1928] reported that pigeons on a diet deficient in the vitamin B complex required for weight recovery a water-soluble, heat-labile factor. This factor differed from the antineuritic vitamin B_1 and the alkali-stable rat factor B_2. Their belief in the existence of this factor was based on the observation that the addition of a special activated fullers' earth preparation of vitamin B_1 cured polyneuritis and checked the decline in weight of birds on deficient diets but failed to restore weight even when large doses were administered. Autoclaved yeast given as a further supplement had no effect, whilst dried yeast produced a gain in weight. Substitution of the synthetic diet for whole wheat also caused a similar increase. The factor postulated to explain these results was designated vitamin B_3.

Confirmation of the views of Williams and Waterman came from several sources. Randoin and Lecoq [1926] had previously stated that pigeons required in addition to vitamin B_1 a growth-promoting factor which was destroyed by autoclaving in an alkaline medium. In discussing vitamin B_3, Lecoq [1931] expressed the opinion that it was identical with the nutritional factor of Randoin and Lecoq. Carter *et al.* [1930] produced evidence which suggested that the pigeon needed, besides vitamin B_1 and B_3, a third alkali-stable factor, vitamin B_5. At the same time, they supported the claims of Williams and Waterman by showing that amounts of vitamin B_1 as large as 20 pigeon day doses caused no gain in weight in birds on polished rice. The possible existence of some such factor as that postulated by these workers needs consideration since it offers an explanation of the fact that quite large doses of concentrates of vitamin B_1 are required together with vitamin B_3 to cause weight increase. Further, Eddy *et al.* [1930] thought that the factor B_3 was essential for the normal growth of the chick as well as the pigeon. They also demonstrated the extreme lability of vitamin B_3 to heat and alkali.

Williams and Eddy [1931] jointly stated that vitamin B_3 was nothing more than an abundant supply of vitamin B_1, ten to twenty times the antineuritic dose. Nevertheless, they could not be certain that their purest preparation of vitamin B_1 was free from B_3. Their opinion was supported by the work of Morris [1933]. She showed that the properties of vitamin B_3 were very similar to those of vitamin B_1 and that pigeons on a diet of autoclaved whole wheat gained in weight when given large doses of vitamin B_1.

In view of the statement of Williams and Eddy, it was considered desirable to report such evidence as we had obtained in an attempt to elucidate the problem of vitamin B_3. The results suggest that pigeons utilise in addition to the vitamin B_1 a factor for weight restoration. We found that supplements of large

doses of vitamin B_1 concentrate given to birds on a polished rice or autoclaved whole wheat diet had no effect upon their weight. Moreover, it was possible to prepare a concentrate of vitamin B_3 which retained its antineuritic curative properties when its weight-restorative potency had been destroyed. Preliminary experiments tend to indicate that the vitamin B_3 effect is not to be attributed to an insufficiency of protein in the basal diet.

Method of assay.

The adult pigeons, used in this investigation, were fed on polished rice, autoclaved at 15 lbs. for 3 hours to destroy completely vitamin B_1. They were weighed every alternate day at 4 p.m. Twice weekly, they were given by mouth 8–10 drops of cod-liver oil as a supply of vitamins A and D. It has been stated that pigeons do not require vitamin C [Sugiura and Benedict, 1923], which is supported by the fact that vitamin C concentrates prepared from lemon juice as a source of vitamin B_3 had little effect. On the diet, the birds dropped to 60–70 % of their maximum weight in a space of 14–21 days which approximates to the length of the storage period of the B_3 factor. To ensure the depletion of the factor, the birds were taken to maximum weight on whole wheat or by additions of 1 g. dried yeast or 3 g. wheat germ daily and then dropped a second time on polished rice. They were now given by mouth 3 doses of vitamin B_1 daily in the form of an alcoholic concentrate prepared according to the method of Kinnersley and Peters. This alcoholic concentrate is considered to contain, together with the antineuritic vitamin B_1, the factor B_5, the rat factor B_4, and possibly also the factor Y of Chick and Copping [1930]. Throughout the present communication, when speaking of vitamin B_1, we refer to the impure alcoholic concentrate. Under these conditions birds can be maintained at surprisingly constant weight far below their maximum for periods as long as 6 months, the fluctuation in weight being never greater than ± 5 g. This weight maintenance has been observed with 60 birds and amply supports the findings of Carter *et al.* [1930]. Under such treatment the birds develop no symptoms peculiar to vitamin B_3 deficiency. The only ill effects seen so far have been occasional cases of birds showing ruffled feathers, lassitude and loss of appetite.

Concentrates and foodstuffs to be tested can now be given into the crop by pipette. Groups of 5 birds were employed to test any preparation. It is to be noted that although three doses of vitamin B_1 suffice for weight maintenance, experiment shows that at least six are necessary in conjunction with a vitamin B_3 preparation to promote weight recovery.

Results.

The richest sources of the factor B_3 proved to be wheat germ and dried yeast. The effect of these supplements on weight is illustrated in Fig. 1 where it is seen that doses of 3 g. wheat germ and 2 g. dried yeast produce a weight recovery practically equal to that obtained on replacing by whole wheat a diet of polished rice supplemented by vitamin B_1 (Fig. 2). Some gain in weight was caused by egg-yolk and to a smaller extent by malt extract and marmite. No effect was seen with egg-white in doses equal to 1 egg per day, liver powder or milk (50 ml./day) even when the dosage of vitamin B_1 was increased to ten times the antineuritic dose.

The restorative effects on weight of the wheat germ and yeast could not be reproduced by supplying the birds with large doses of the concentrate of Kinnersley and Peters. Doses of vitamin B_1 of forty times the curative amount

have had no influence upon weight. Such doses would presumably contain adequate supplies of the other factors present. A concentrate of vitamin B_2 was prepared from aqueous extract of yeast by lead precipitation. The precipitate was decomposed by warming with sulphuric acid, and the last traces of

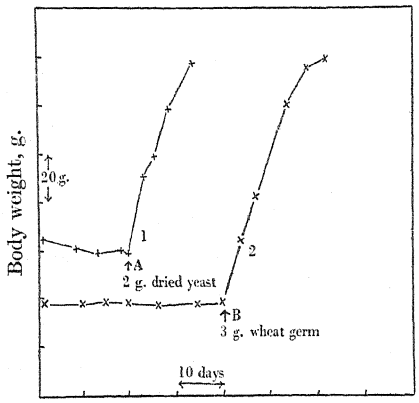

Fig. 1.

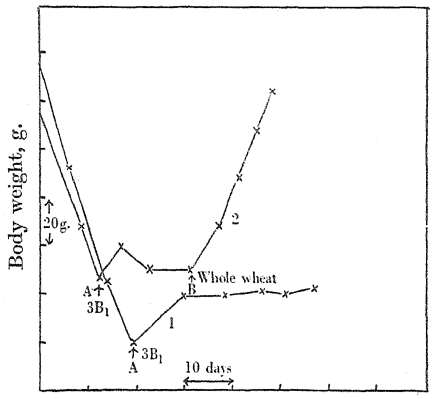

Fig. 2.

Fig. 1. Weight recovery with wheat germ and dried yeast. Typical curves obtained on giving dried yeast (curve 1 A) or wheat germ (curve 2 B) to birds on polished rice + 6 doses vitamin B_1.

Fig. 2. Weight recovery with whole wheat. Curve 1 shows drop in weight of bird on polished rice. At A, 3 doses B_1 are given. Curve 2 shows a similar case. At B, a whole wheat diet is substituted for polished rice + 3 B_1.

lead were removed by H_2S. The solution obtained was inactive as a source of vitamin B_3 in doses four to five times the amount required to cure young rats of dermatitis and to promote growth of 15–20 g./week. From these experiments, it was concluded that none of the factors investigated was responsible for the vitamin B_3 effect.

Preparation of vitamin B_3 extracts.

Attempts were made to prepare extracts of vitamin B_3 from foodstuffs normal to pigeons' diets—maize, corn, wheat. Aqueous and alcoholic extracts were inactive as supplements. Similar extracts of yeast were likewise without activity. It was found that yeast extracted with 97 % alcohol at 40° retained only 50 % of the original vitamin B_3 activity. Slight activity was shown by extracts of grains prepared by means of acidic solvents. These observations led to the conclusion that vitamin B_3 was being held in some form of chemical combination insoluble in the usual solvents. More drastic chemical treatment was therefore attempted to liberate the factor, the most successful being acid hydrolysis. Potent extracts were obtained by hydrolysis of wheat germ and dried yeast.

The wheat germ extract was prepared in the following manner. Wheat germ was extracted three times at 60° with 97 % alcohol and dried in air. Batches of 75 g. were hydrolysed with 300 ml. N H_2SO_4 by vigorous boiling under reflux for 6 hours. The mixture was filtered while hot and cooled. The main bulk of the sulphuric acid was removed by the addition of baryta or barium carbonate until the p_H was about 2. Before dosing, these concentrates were brought with NaOH to p_H 6·8. Given in doses equivalent to 3 g. of wheat germ, the hydrolysate caused an immediate increase in weight in pigeons (Fig. 3).

The properties of the extracts are as follows: There is present little or no protein matter, since the usual protein reagents, trichloroacetic acid, alcohol, sodium tungstate, ammonium sulphate and sodium sulphate caused negligible precipitation under varied conditions. Amino-acids are present together with some reducing substances. The extracts contain vitamin B_1. Tests on two groups of 5 birds in head retraction showed that on an average, there are 4–5 doses of B_1 in one dose of hydrolysed wheat germ. The peculiar property of the extracts is the rapid loss in vitamin B_3 activity on standing at room temperature or in cold store for 6–8 days. In Fig. 3, it is seen that a rapid rise in weight occurs with a freshly prepared hydrolysate followed by decline or maintenance in weight. The addition of another fresh extract renews the weight increase.

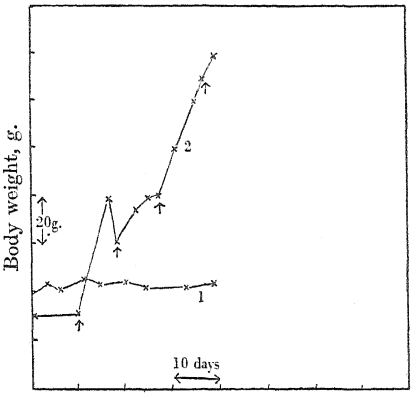

Fig. 3. Weight recovery with hydrolysed wheat germ. Curve 1, control bird on polished rice + 6 doses B_1. Curve 2, bird receiving in addition dose of hydrolysed wheat germ ≡ 3 g. wheat germ daily. The arrows indicate fresh preparations of hydrolysate.

It seemed probable that the inactivation of vitamin B_3 arose from oxidation. Such a view was supported by the fact that when air was drawn through the hydrolysed wheat germ preparations at p_H 2 for 1 hour almost complete loss of activity was observed. Similarly, after treatment with hydrogen peroxide under the same conditions, the extracts produced no effect on weight. Further, the loss in activity can, to some extent, be inhibited by bubbling nitrogen through the mixture during hydrolysis and storing the preparations in nitrogen or *in vacuo*. By such procedure, the activity can be preserved for 10–12 days. Other attempts at preservation with carbon dioxide atmospheres, however, caused inactivation. Little success was obtained with the addition of reducing substances such as sulphite as preservatives. The vitamin B_1 content is unimpaired by oxidation with air; tests on a group of 5 polyneuritic birds revealed that the original 4–5 doses of vitamin B_1 per dose of hydrolysate were retained.

Attempts at fractionation of vitamin B_3 preparations.

The very labile character of vitamin B_3 has rendered difficult attempts to prepare more potent extracts. As mentioned above, protein precipitants are useless. Fractionation with phosphotungstic acid and mercury salts have also proved unsatisfactory. With lead acetate, on some occasions, at least 50 % of the activity has been precipitated but the results have been so variable that it was impossible to use this reagent. It can be concluded from these experiments that the factor B_3 behaves quite differently from vitamin B_1.

Behaviour of vitamin B_3 towards heat.

Eddy *et al.* [1930] considered that vitamin B_3 was extremely heat-labile, being destroyed at temperatures above 60°. On the other hand, Carter *et al.* [1930] state that the vitamin B_3 potency of whole wheat is not completely destroyed after 5 hours' steaming. The conclusions reached by these two groups of workers are directly contradictory. From our experiments, in its behaviour

towards heat the factor B_3 is somewhat similar to vitamin B_1 in that it is more susceptible to moist than to dry heat. We have shown that vitamin B_3 resisted boiling with N H_2SO_4 for 6 hours, which indicates a stability greater than would be expected from the results of the American workers. We have also observed that complete destruction of the factor occurred on autoclaving whole wheat, wheat germ and yeast at 120° for 3 hours while heating these materials at 120–160° for 2 hours hardly impaired the activity. The divergent results of different investigators suggest that the stability of vitamin B_3 is probably dependent upon its state of combination in the foodstuff.

The relation of protein to the vitamin B_3 effect.

Considerable criticism has been levelled at the postulation of factors other than vitamin B_1 being essential for maintenance nutrition of pigeons, on the basis that the experimental diets were not always adequate in respect to other nutrients. The use of polished rice as a basal diet has been particularly condemned. The question of an insufficiency of mineral salts and other vitamins does not arise in our experiments. Further, the caloric requirements are satisfied. Food intakes were measured, and it was found that a pigeon on a polished rice diet receiving 6 doses daily of vitamin B_1 ingested 20 g. daily, the caloric value of which is 71. On a whole wheat diet, which supplies all the essentials for pigeon nutrition, a bird will consume 25 ± 5 g. daily; caloric value 86 ± 17. Sugiura and Benedict [1923] found that pigeons could be maintained on 20 g. of a synthetic diet of a caloric value of 70. The discrepancies in the caloric values of the amounts of different diets consumed daily are hardly sufficient to explain the failure of vitamin B_1 concentrates to restore weight in pigeons on polished rice.

Since the low protein content of polished rice might be responsible for the failure, experiments were made in which protein was given to birds. 2 g. of caseinogen were given daily to birds on rice supplemented by 6 doses of vitamin B_1. No response was observed. When, however, the dosage of vitamin B_1 was doubled, rapid increase in weight was seen in the first 4–7 days of the experiment followed by large fluctuations in the next 7 days. Two groups of 5 birds were used in the experiments and in no case was a rise to maximum weight obtained. In another series of experiments, 2 g. caseinogen were given to birds receiving hydrolysed wheat germ equivalent to 3 g. wheat germ daily in addition to the vitamin B_1 supplement. Complete recovery to maximum weight was obtained (Fig. 4). In Fig. 4, it is shown that the weight increase ceased when the vitamin B_3 concentrate lost its activity.

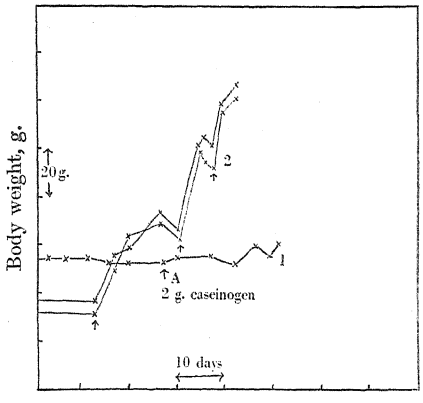

Fig. 4. Caseinogen supplements. Curve 1, control bird on polished rice + 6 doses B_1 daily. At A, 2 g. caseinogen given daily. Curve 2, birds on polished rice + 6 doses B_1 + hydrolysed wheat germ $\equiv$ 3 g. wheat germ + 2 g. caseinogen daily. The arrows indicate fresh preparations of hydrolysate given when decrease in weight occurred with loss in activity of vitamin B_3.

Morris [1933] has tried to overcome the difficulty of the insufficiency of protein by the use of autoclaved whole wheat as a basal diet. She obtained recovery in weight by giving doses of vitamin B_1 roughly six to twelve times the dose curative of polyneuritis, and concluded that

the vitamin B_3 effect was probably a function of a large quantity of vitamin B_1. Our experience differed from that of Morris. A group of 10 birds was placed on whole wheat autoclaved at 120° for 3 hours, and when they had dropped to 60–70 % of their maximum weight, they were given 6 of doses vitamin B_1 daily. After slight fluctuations, the weights of the birds became constant. No recovery in weight was observed on increasing the amount of vitamin B_1 to 30 pigeon day doses. The disagreement of the results of our experiments on caseinogen supplements with those of Carter [1934] and the failure to confirm Morris on autoclaved wheat are possibly connected with fuller depletion of factors in the preparation of the birds used here, but the matter requires further investigation.

Discussion.

From the experiments so far made, it appears that some factor is required for weight restoration by a pigeon on a diet of polished rice supplemented by an alcoholic concentrate of vitamin B_1. The nature of this factor is doubtful. Our results certainly fail to substantiate the statement of Williams and Eddy [1931] and of Morris that vitamin B_3 is merely an abundance of vitamin B_1, many times the dose curative of polyneuritis. The fact that pigeons rise rapidly in weight when removed from polished rice to whole wheat is difficult to reconcile with this view. The daily intake of a bird on wheat would scarcely provide it with such large doses of vitamin B_1. Hydrolysed wheat germ contains a factor which can be distinguished from vitamin B_1 by its instability to oxidation. The mode of preparation of the extract together with its properties suggest that the factor may be an amino-acid. It may, in fact, be one or more of the known amino-acids which, owing to the nature of the extract, break down readily on oxidation. But no evidence has yet been obtained by us in experiments in which known amino-acids such as histidine (20 mg. given daily to 2 birds), tryptophan (20 mg. daily to 2 birds), or cystine (50 mg. daily to 3 birds), present in negligible amounts in polished rice, have been administered together with vitamin B_1 that such a view is correct. In fact, the experiments with caseinogen supplements suggest that the vitamin B_3 effect does not arise from a faulty basal diet.

Summary.

1. Supplements of 20–30 doses of vitamin B_1 fail to restore the weight of pigeons on a polished rice or autoclaved whole wheat diet.

2. Extracts can be prepared from wheat germ which restore the weight of pigeons on polished rice supplemented by vitamin B_1. These extracts are inactivated by oxidation.

3. Birds on polished rice + 6–12 doses daily of vitamin B_1 fail to recover weight when given 2 g. caseinogen daily.

4. It is concluded that the pigeon requires in addition to vitamin B_1 a factor to restore weight.

I wish to express my thanks to Prof. Peters for his advice and criticism throughout this work and to the Department of Scientific and Industrial Research and the Medical Research Council for grants.

REFERENCES.

Carter (1934). *Biochem. J.* **28**, 933.
—— Kinnersley and Peters (1930). *Biochem. J.* **24**, 1832, 1844.
Chick and Copping (1930). *Biochem. J.* **24**, 1764.
Eddy, Gurin and Keresztesy (1930). *J. Biol. Chem.* **87**, 729.
Lecoq (1931). *J. Biol. Chem.* **91**, 671.
Morris (1933). Dissertation. New York.
Randoin and Lecoq (1926). *Compt. Rend. Acad. Sci.* **182**, 1408.
Sugiura and Benedict (1923). *J. Biol. Chem.* **55**, 33.
Williams and Eddy (1931). Carnegie Year Book.
—— and Waterman (1928). *J. Biol. Chem.* **78**, 311.

CXXXIII. MAINTENANCE NUTRITION IN THE PIGEON AND ITS RELATION TO HEART BLOCK.

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In a previous paper [Carter, 1930] it was shown that pigeon heart block which develops on a diet of polished rice could be cured if the birds received daily rations of whole wheat constituting not less than about 60 % of the total food intake. Vitamins B_1 and B_2 and the fat-soluble group of vitamins were found to be ineffective in restoring normal heart function when administered as supplements to the diet of polished rice. Heart block in the pigeon is therefore a condition of dietary origin. The question remains whether it is due to the deficiency of a specific factor as is the case in polyneuritis, or whether it arises from a deficiency of one or more of those factors which contribute to maintenance nutrition at maximum weight.

In some experiments to be described it will be shown that pigeons previously depleted on a diet of polished rice can, in a majority of cases, be restored to maximum weight by appropriate supplements to this diet without inducing any recovery of normal heart function. Substitution of an extract of hydrolysed wheat germ for the supplements results in a complete recovery from heart block without further significant change in weight. It will be further shown that birds previously brought to maximum weight on whole wheat can be maintained at this level on a diet which fails to protect them from the onset of heart block. The fact, previously recorded, that birds depleted on polished rice, when fed on whole wheat generally recover normal heart rhythm at a time when restoration of body weight is far from complete, has been confirmed. Finally the protein intake of birds on a polished rice diet has been examined, and its significance in relation to the problem of pigeon nutrition is considered.

Method.

Birds under test were kept singly in small cages in order to obtain measurements of the food intake. Observations on body weight were made at the same time each morning in order, as far as possible, to avoid fluctuations due to variations in the amount of food present in the crop. Heart rate and rhythm were noted at frequent intervals.

The source of vitamin B_1 employed was a yeast concentrate prepared according to the method of Kinnersley and Peters [1927] by the extraction of activated norite charcoal with 50 % acid alcohol[1]. In all cases cod-liver oil has been given at regular intervals. Caseinogen (Glaxo, "Vitamin B-free") was

[1] This partially purified concentrate may contain, in addition to vitamin B_1, another factor, vitamin B_5, which has been suggested as being necessary for the pigeon [Carter *et al.*, 1930].

extracted continuously in a Soxhlet apparatus with 90 % alcohol for 48 hours and dried. It has been assumed in this paper that caseinogen treated in this manner provides a source of protein free from possible contaminating substances which might influence the nutrition of the pigeon. Before administration by pipette portions of 2 g. were weighed out, suspended in 20 ml. Ringer solution and brought to p_H 7·0 by addition of caustic soda. Hydrolysed wheat germ was prepared by heating for 3 hours with N hydrochloric acid and filtering hot on a Büchner funnel. This method, which differs from that employed by O'Brien [1934], was used daily to prepare the extract in order to avoid the possibility of its deterioration on standing in air.

Experimental.

The earlier experience of the effect of marmite, which in daily doses of 1·5 g. administered over periods of 30 days and upwards showed curative activity in regard to heart block in a certain proportion of cases, prompted a hope that the factor concerned might be fractionated from yeast. The negative results which have invariably been obtained with vitamin B_1 concentrate suggested that preparations from the precipitate which appears on treatment of an aqueous yeast extract with basic lead acetate might be effective. The results have unfortunately been variable and do not at present justify detailed consideration.

The lead precipitate, after treatment with sulphuric acid followed by hydrogen sulphide to remove all traces of lead, when administered to pigeons fed on polished rice is entirely without influence either in preventing decline in weight or in improving the heart condition. In an experiment in which this extract was supplemented by daily doses of an antineuritic concentrate together with 2 g. caseinogen 2 birds showed restoration of normal heart function within 20 days, while in another bird definite improvement though incomplete cure was noted. In all three cases there was gradual recovery over a period of 50 days to maximum weight. Repetition of this experiment with similar treatment resulted in complete cure of heart block in one bird, partial recovery in a second, and no improvement in a third. Restoration to weight maximum was observed in two of these birds while in the third the rise of weight, though substantial, failed to reach that obtained on whole wheat.

These preliminary experiments made it important to ascertain whether the induction and cure of heart block could be dissociated from factors associated with maintenance or restoration of normal body weight.

Effect of yeast concentrate and caseinogen on pigeon nutrition.

A group of 8 birds was placed on a diet of polished rice for periods varying from 12 to 24 days. During this time the usual fall of weight and onset of heart block occurred. In one case acute polyneuritis developed before treatment was commenced. There is at present insufficient evidence to indicate whether variation in the duration of the depletion period has any influence on the subsequent response. A control group of 3 birds was given daily doses of a vitamin B_1 concentrate in amounts equivalent to 12 units daily.

Table I and Fig. 1 show that the progressive decline in weight is arrested and that there is some rise which is less marked than that observed with birds receiving in addition a daily supplement of 2 g. caseinogen. This confirms previous experiments in which maximum weight has not been fully restored by supplements of yeast concentrate or marmite to the basal diet of polished rice.

Table I. *Effect of polished rice and 12 units vitamin B_1.*

Bird	Maximum weight g.	Weight after depletion on polished rice	Duration of depletion days	Final weight	Duration of curative period days
880	396	287	17	338	28
110	400	250	24	260	28
111	360	218	24	255	28

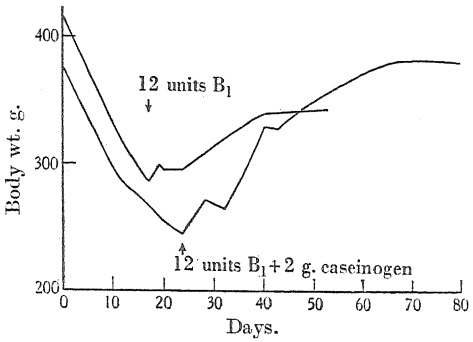

Fig. 1.

Table II records similar data for those birds on polished rice receiving daily 12 units of vitamin B_1 and 2 g. caseinogen.

Table II. *Effect of polished rice supplemented by 12 units vitamin B_1 and 2 g. caseinogen.*

Bird	Maximum weight g.	Weight after depletion on polished rice	Duration of depletion days	Final weight	Duration of curative period days
401	324	268	12	325	59
357	376	247	24	380	53
722	468	286	24	398	57
622	392	268	15	376	54
485	390	285	15	380	54

It will be seen that of these birds only one, No. 722, failed to reach maximum weight in the course of the experiment. In this respect their behaviour is in agreement with that of the birds in the earlier experiments.

In another experiment 2 birds, previously brought to maximum weight on mixed corn, were placed on polished rice supplemented by 2 g. caseinogen and 12 units of vitamin B_1 daily. After an initial fall of weight of 28 and 16 g. respectively during the first 4 days there was almost no change throughout the experimental period which lasted for 37 days (Table III).

Table III.

	Day	Bird 622	Bird 485
Initial maximum weight	0	418	434
Weight after commencement of experimental diet	4	402	406
	10	405	411
	20	399	404
	30	390	396
	37	398	401

The rather unexpected result of these experiments raises a number of points of interest. In the first place the rise of weight to maximum is notably slow in comparison with the rise seen on whole wheat. Of the birds referred to in Table II full weight was not attained in two cases until the 40th day while in another it was delayed until the 59th day. This is about four times as slow as in the case of whole wheat. The fact that the weight rise is so gradual under these conditions raises the possibility that experiments of short duration may lead to misleading conclusions.

While the attainment of maximum weight upon a given diet may, subject to the possibility of storage of essential factors during the initial depletion period, be justifiably held to indicate that such a diet is "adequate" for full nutrition it does not necessarily follow that it contains a satisfactory balance of essential dietary constituents such as is present in whole wheat. In two respects a diet of polished rice may be ill-balanced. In 4 birds it was found that daily administration of 6 units of vitamin B_1 was inadequate with caseinogen, whereas an increase of dosage to 12 units sufficed to promote slow continued rise of weight. This large daily requirement of vitamin B_1 may be more apparent than real since the concentrate in which it is administered is also the source of vitamin B_5, another essential dietary constituent for maintenance in the pigeon, and it is possible that the latter may here be a limiting factor. On the other hand, as suggested by Williams and Eddy [1931], there may be a real difference in the requirement of vitamin B_1 for cure of polyneuritis and for maintenance of full nutrition. Morris [1933] claims that, with a basal diet of autoclaved wheat, the dose of a vitamin B_1 concentrate necessary to effect complete weight recovery after depletion exceeds the dose required for cure of polyneuritis by an amount which varies from six to sixteen times with different types of yeast concentrate. On the other hand where the basal diet is one of polished rice full recovery does not occur even if as much as 30 curative doses of vitamin B_1 are administered daily [Carter *et al.*, 1930]. The difference in the nature of the basal diet is important. The factor which appears to limit weight recovery on polished rice supplemented by a yeast concentrate is a relative deficiency of protein. Moreover a difference in the type of protein available on diets of wheat on the one hand and polished rice supplemented by caseinogen

Table IV. *Average daily intake.*

	Bird 880			Bird 110			Bird 111		
	Total cals.	Protein g.	Days on test	Total cals.	Protein g.	Days on test	Total cals.	Protein g.	Days on test
Wheat	79·23	2·50	9	72·34	2·29	11	80·95	2·56	5
Rice with 5 units vitamin B_1	71·42	1·18	5	—	—	—	—	—	—
Rice with 10 units vitamin B_1	83·20	1·37	10	60·70	1·00	10	59·27	0·98	10
Rice with 12·5 units vitamin B_1	85·34	1·41	11	55·34	0·92	9	71·42	1·18	10

	Bird 622			Bird 401		
	Total cals.	Protein g.	Days on test	Total cals.	Protein g.	Days on test
Wheat	89·57	2·83	6	82·7	2·61	6
Rice with 2 g. caseinogen, and 12 units vitamin B_1	85·0	3·15	52	87·8	3·18	13

on the other may perhaps contribute to the difference in the time required for weight recovery in the two cases. Table IV shows the calory and protein intake for birds on diets of polished rice supplemented by a yeast concentrate, with and without addition of caseinogen.

Birds 110 and 111 show some diminution in their daily calory intake as compared with that on whole wheat, whereas in the case of bird 880 this appears to be satisfactory. Sugiura and Benedict [1923] found, in experiments in which pigeons were forcibly fed on a diet containing 22 % caseinogen, that a daily intake of about 70 calories sufficed for normal growth and maintenance at full weight.

There is however in all birds on a rice diet a substantial diminution of the daily protein intake. On the other hand birds 622 and 401, which rose to their maximum initial weight on a rice diet to which were added 2 g. caseinogen in addition to the yeast concentrate, show daily calory and protein intakes which compare favourably with the intakes on whole wheat.

The biological value of the protein of polished rice has been regarded by Osborne *et al.* [1915] as high, but on the other hand its protein content is only about 50 % of that of whole wheat. There is at present no information as to the minimum protein requirement for the pigeon at maximum weight and the figures for protein intake on a polished rice diet do not therefore prove that this intake is inadequate. However, the fact that an additional protein supplement in the form of caseinogen to the basal diet enables the bird to regain full weight suggests that insufficient polished rice is consumed to meet its protein requirement. In this connection the observations of Aykroyd [1930], on the significance of partial protein deficiency in human beri-beri, may be mentioned.

It is desirable at this stage to define the sense in which the term "maximum weight" has been used in the preceding experiments. It is not here intended to imply that maximum weight represents some fixed and constant level of nutrition for any particular bird, since the body weight at any given moment is determined not only by the nature of the diet which it is receiving but also by its past dietary history and doubtless by other factors. The birds, before experiment, are placed on mixed corn for some weeks, and during this time come into equilibrium at a weight which has been here termed "maximum." If, after a period on an experimental diet having polished rice as its basis, the birds are returned to whole wheat the new weight which is established is not necessarily identical with the former maximum. In some cases, examples of which will be seen in experiments with wheat germ, the new weight level somewhat exceeds the initial "maximum." On the other hand a number of cases have been met with during the course of work in this laboratory in which whole wheat or corn fails to restore the full original weight level. It is not the purpose of this paper to discuss the factors underlying these variations in maximum weight. The facts must be borne in mind in assessing the significance of the experiments with caseinogen which have been described.

Effect of vitamin B_1 and caseinogen on pigeon heart block.

In the experiments described in the preceding section repeated observations were made on the condition of the heart. At the end of the initial depletion period on polished rice irregularity of rhythm and bradycardia, which in some cases was very marked, were observed. For example bird 722, after a depletion period of 24 days, showed a fall of heart rate from 200 per minute to 66 per minute.

It has been frequently observed that some rise of rate is associated with rise of body weight though the persistence of irregularity of rhythm shows

that the underlying disorder of the vagus centres remains. In all the birds whose weights are recorded in Table II heart block persisted through the entire period of the experiment in spite of a weight rise to maximum in four of them. Table V shows records of the heart in two typical birds of this group.

Table V.

Days on experimental diet	Heart rate and rhythm			
	Bird 722		Bird 357	
0	66	Regular	112	Dropped beats
4	144	Dropped beats	144	,,
9	132	,,	164	,,
18	144	,,	148	,,
26	128	,,	164	,,
38	148	,,	156	,,
54	128	,,	160	,,

In the experiment in which birds, having attained maximum weight on a diet of mixed corn, were maintained at this weight on a diet of polished rice supplemented by caseinogen and vitamin B_1 concentrate, heart block developed at the 8th and 15th days respectively and persisted through the experiment lasting 37 days.

These experiments show that normal nutrition as judged by weight tests can be established in conjunction with a dietary deficiency which is revealed by the presence of cardiac arhythmia. Experiments now to be described show that this deficiency can be supplied by a constituent of wheat germ.

Effect of wheat germ on pigeon heart block.

Table VI shows the effect of administration of wheat germ hydrolysed for 3 hours in association with 12 units of vitamin B_1 concentrate.

Table VI.

Bird	Before administration of wheat germ			After administration of wheat germ (6 g.)			Duration of treatment days	Initial maximum weight g.
	Heart rate		Weight g.	Heart rate		Weight g.		
622	140	Dropped beats	376	152	Regular	427	23	392
485	100	,,	380	176	,,	401	16	387
722	160	,,	398	174	,,	423	13	468
357	160	,,	380	192	,,	399	14	376
				Wheat germ 4 g.				
940	124	,,	253	160	Dropped beats	320	39	338
				Wheat germ 8 g.				
				176	Regular	330	4	—
				Wheat germ 4 g.				
921	132	,,	282	160	Regular	313	20	368
				Wheat germ 6 g.				
44	116	,,	299	176	Regular	380	19	362

The first 4 birds had previously received caseinogen and the yeast concentrate with the results already described. Wheat germ extract in doses equivalent to 6 g. cleared up the cardiac irregularity in each case after an interval of 13–23 days. This curative interval is considerably longer than that usually observed with whole wheat, but it should be noted that the method of preparing the extract involves considerable losses, and the actual amount administered

is probably substantially less than the equivalent of 6 g. of dry wheat germ. It will be seen that in three of these birds there is a rise in weight which somewhat exceeds the initial weight maximum. The significance of this fact has already been discussed. The remaining birds received wheat germ extract immediately following the depletion period. Birds 921 and 940 received the equivalent of 4 g. of the extract, and in the case of the latter this dose failed to improve the cardiac condition in a period of 39 days. An increase in the dose to the equivalent of 8 g. resulted in complete recovery of the heart in 4 days.

A final experiment in which a group of birds, after depletion on polished rice, were returned to whole wheat, provides further evidence for the view that restoration of heart block can be effected independently of the state of nutrition of the bird as judged by its body weight. Table VII shows that restoration of the heart occurred within 2–5 days after commencement of a whole wheat diet, and at a time when recovery of body weight was still incomplete.

Table VII.

Bird	Maximum weight g.	Weight after depletion on rice g.	Weight at time of recovery of heart g.	Heart rate after depletion on rice	Heart rate after recovery on wheat	Time for recovery of heart days
142	488	317	400	100 Dropped beats	156 Regular	2
912	346	229	293	128 ,,	240 ,,	5
157	450	342	419	80 Regular	200 ,,	5
23	384	251	310	108 Dropped beats	184 ,,	3

SUMMARY.

The experiments outlined in this paper provide evidence for the view that one factor limiting the restoration, after depletion, of full weight of pigeons on a diet of polished rice supplemented by a yeast concentrate is an inadequate allowance of protein in the diet. Where this partial deficiency is made good by the addition of caseinogen full restoration of weight usually occurs. It is not finally proved that caseinogen, purified in the manner described, provides no factor other than protein, and it would therefore be premature to draw conclusions from these experiments as to the necessity for vitamin B_3 in pigeon nutrition. The evidence does however indicate that maintenance of, or recovery to, full weight may be compatible with the persistence of a cardiac arhythmia of dietary origin. This dietary constituent can be extracted from wheat germ, and its administration in amounts equivalent to 6–8 g. leads to complete restoration of function.

I desire to record my sincere thanks to Prof. Peters for his continued interest and advice.

REFERENCES.

Aykroyd (1930). *J. Hygiene*, **30**, 357.
Carter (1930). *Biochem. J.* **24**, 1811.
—— Kinnersley and Peters (1930). *Biochem. J.* **24**, 1832.
Kinnersley and Peters (1927). *Biochem. J.* **21**, 778.
O'Brien (1934). *Biochem. J.* **28**, 926.
Osborne, Van Slyke, Leavenworth and Vinograd (1915). *J. Biol. Chem.* **22**, 259.
Morris (1933). Dissertation.
Sugiura and Benedict (1923). *J. Biol. Chem.* **55**, 33.
Williams and Eddy (1931). Carnegie Report.

CXXXIV. THE COLLOIDAL PHOSPHATE OF MILK.

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The presence of insoluble, or rather colloidal, calcium phosphate in milk has been recognised for a long time; but despite numerous researches both into the type of phosphate present and the nature of its association with other colloids, it cannot yet be said that either of these matters has been definitely cleared up. To judge from the literature, opinion seems to be still divided, firstly, as to whether the phosphate is secondary or tertiary, or a mixture of the two, and secondly, as to whether it is in chemical combination with calcium caseinogenate or merely protected by it. It is hoped in the present paper to present some additional evidence on these points, which appears to favour the view that the phosphate is mainly tertiary and is present in intimate union with the caseinogenate.

Hammarsten [1879] supposed that calcium phosphate is held in solution in milk by calcium caseinogenate, and Lehmann [1894] regarded the two as united to form a double salt in which the phosphate is the tricalcium salt. Lindet's views [1912] appear to be similar. Söldner [1888] concludes that both di- and tri-calcium phosphate are present in milk, the former in somewhat the greater amount, and apparently rejects the hypothesis of a chemical union between these substances and caseinogen. Hoffman and Gortner [1923] and Porcher [1930] also consider the phosphate to be a mixture of the same two salts and their relation to the protein to be solely one of colloidal protection. Van Slyke and Bosworth [1914, 2], while accepting and apparently verifying, by their experiments on the centrifugal separation of milk, the merely protective rôle of the caseinogenate to calcium phosphate, consider the latter to be the di-calcium salt exclusively—an opinion apparently shared by Palmer [1924], Grimmer [1926], Wright [1928] and many others. On the other hand, György's experiments [1923] on the pancreatic digestion of milk and of artificially prepared caseinogen-calcium phosphate complexes led him back to the earlier view that some type of definitely chemical combination exists between calcium phosphate and the protein. Recent work by Kometiani [1931] on heated milk, which will be referred to more fully later, also supports this view.

In the course of some work in another connection [1932], the present writer noticed (*a*) that the formaldehyde titration of milk protein was much lower in the presence of colloidal calcium phosphate than in its absence, and (*b*) that the titratable acidity of milk was greatly diminished on addition of a sufficient quantity of a soluble oxalate. The first observation seemed to bear the interpretation, then stated, that the colloidal phosphate and calcium caseinogenate were chemically combined; and the second, which was not indeed new, but was susceptible of a different explanation [*cf.* Pyne and Ryan, 1932] from that given

by Van Slyke and Bosworth [1914, 1], suggested that the phosphate was largely tertiary.

The present paper contains a development of these observations and some others, which appear to confirm these views.

A. *Relation of calcium phosphate to calcium caseinogenate.*

In the previous paper [1932] it was shown that addition of a soluble oxalate (4 % of the saturated potassium salt) to milk increased its formaldehyde titration by about 11 % on the average. For some artificially prepared caseinogen-calcium phosphate complexes in which about one-half of the total calcium was combined with phosphoric acid, the percentage increase in formaldehyde titration was somewhat greater on oxalation, *viz.* about 16 %. (The oxalate is regarded as dissolving the union between the caseinogenate and the phosphate.)

Somewhat similar results have since been obtained with other complexes of the same type, *e.g.* a caseinogen-calcium carbonate, and also with a caseinogen-calcium phosphate complex to which gelatin had been subsequently added. On the other hand, where colloidal calcium phosphate was protected by gelatin, the addition of oxalate did not appreciably affect the formaldehyde titration, and this held even when calcium caseinogenate, subsequently added, was present in the mixture at the time of oxalation and titration. A few typical results are given in Table I.

The caseinogen-carbonate complex was prepared, following Porcher [1930], by bubbling carbon dioxide at ordinary temperature through an alkaline solution of calcium caseinogenate (3·7 % CaO) until decidedly turbid and then removing the excess carbon dioxide by a current of air; the gelatin-calcium phosphate colloid was obtained according to the directions of De Toni [1921].

Table I.

Solution	% total protein	Formaldehyde value ml. *N* alkali per 100 ml. solution	
		With oxalate	Without oxalate
Caseinogen-calcium phosphate complex	2·3	1·30	1·15
,, carbonate complex	2·0	1·14	0·98
Gelatin-calcium phosphate, Complex A	4·5	1·39	1·37
,, ,, B	—	1·32	1·31
,, ,, C + calcium caseinogenate (2·5 %)	4·75	2·05	2·07

It is clear that protection of colloidal calcium phosphate (and carbonate) by calcium caseinogenate is quite different in type from protection by other proteins, *e.g.* gelatin. The marked difference is most simply explained by assuming that in the first case, where the properties of the protein are considerably modified by this "protection," the union is more definitely chemical. The potentiometric titration of the complex described later in this paper also favours this view.

In this connection the experiments of Kometiani [1931] on heated milk are of interest. This author attributes some at least of the increased titratable acidity that results from heating to a partial separation of calcium phosphate from its caseinogenate complex, thereby increasing the number of free carboxyl groups in the protein and thus its buffer value and titratable acidity. In apparent confirmation of this view he finds that the formaldehyde titration of milk increases on heating, the increase reaching its highest value of about 15 % on heating milk for half-an-hour at 120°. It is remarkable that a difference of

much the same order is shown (*cf.* Table I) for the formaldehyde titration of calcium caseinogenate according to whether it is associated with calcium phosphate or not.

These findings favour then the views of György, *i.e.* that the relation of calcium phosphate to the caseinogenate is one of chemical union and not merely of colloidal protection. As to the type of union between them, its rapid dissolution under the action of oxalate suggests that the common bond is one or more calcium atoms, and that the complex is thus a double salt of caseinogenate and phosphate. The marked shift in the reaction of the complex towards alkalinity on addition of potassium oxalate, a matter which will be discussed more fully later, makes it probable that the phosphate liberated is largely K_3PO_4 arising from the tertiary calcium salt. The complex should on these views be essentially a double salt of calcium caseinogenate and tricalcium phosphate, and its primary interaction with oxalate might be represented schematically by:

$$(NH_2)_p\text{—R}\begin{matrix}\diagup(\text{COOH})_l\\ \text{—(COOCa}')_m\\ \diagdown(\text{COO.Ca.PO}_4\text{.Ca})_n\end{matrix} + \tfrac{3}{2}n.K_2\overline{Ox} = (NH_2)_p\text{—R}\begin{matrix}\diagup(\text{COOH})_l\\ \text{—(COOCa}')_m\\ \diagdown(\text{COOK})_n\end{matrix} + \tfrac{3}{2}n.Ca\overline{Ox} + n.K_3PO_4$$

where Ca′ represents for convenience a half-atom of calcium. It is of course not intended to suggest that the phosphate complex should be a definite individual salt, but only a certain type of compound in which however the proportion of phosphate can be varied greatly according to the method of preparation. The evidence in favour of the presence of the elements of tricalcium phosphate in this complex will now be considered more fully.

B. *Nature of the colloidal phosphate.*

Ignoring the small amount of colloidal magnesium phosphate which is present in milk in somewhat the same form, no doubt, as the corresponding calcium phosphate, and regarding the caseinogen-calcium phosphate of milk as essentially a double salt of caseinogenate and phosphate, the exact nature of the latter, whether secondary, tertiary or a mixture of these remains to be determined. In a sense, it may appear immaterial which, for owing to the variation in the calcium content of the caseinogenate with p_H, a compound of, say, dicalcium phosphate and calcium caseinogenate, might be regarded from one point of view as identical with one of tricalcium phosphate and a more acid caseinogenate. For definiteness, however, the colloidal calcium phosphate ingredient of the complex must be considered to be that phosphate which can unite with calcium caseinogenate to form the complex without any considerable change in p_H. This is, in fact, the assumption made by Porcher in his experiments on the synthesis of the complex (see later).

Action of soluble oxalate on milk. The writer and Ryan [1932] have shown elsewhere that addition of a soluble oxalate to milk in sufficient amount (*e.g.* 4 % of a saturated solution of potassium oxalate) reduces the titratable acidity to phenolphthalein by about 1·20 ml. N/100 ml. milk. For whey, on similar treatment, the fall in acidity is about 0·30 ml. N/100 ml., so that the net alkalinity developed by the interaction of oxalate and the caseinogen-calcium phosphate complex of the milk amounts to about 0·90 ml. N/100 ml. The apparent source of most of this alkalinity is K_3PO_4 produced from tricalcium phosphate by the action of oxalate. For solutions of calcium caseinogenate alone develop only very slight alkalinity on addition of oxalate, and any colloidal dicalcium phosphate ($CaHPO_4$) that may be present in the complex will give rise to

K_2HPO_4 which is practically neutral to phenolphthalein[1]. Assuming then that 0·90 ml. *N* represents approximately the amount of acid to convert the tricalcium phosphate of 100 ml. milk *via* K_3PO_4 into K_2HPO_4, the tricalcium phosphate content of milk can be calculated to be about 0·14 g. $Ca_3P_2O_8$/100 ml., containing 0·076 CaO. Söldner [1888] estimates the lime content of the colloidal phosphate to be 0·071 g./100 ml. milk and Porcher and Chevallier [1923] about 0·065 g. The figure obtained from oxalate titration, which represents the lime content of tricalcium phosphate only, does not differ greatly from these values, and the agreement suggests that much or all of the colloidal phosphate of milk is tertiary.

More direct evidence is obtained by comparison of the tricalcium phosphate (calculated from ordinary and oxalate titrations of milk and whey as above) and the total colloidal phosphate for the same sample obtained by analysis. The latter was taken as the difference between the acid-soluble inorganic phosphorus contents of milk and rennet whey determined by the Briggs colorimetric molybdate method and using the technique of Lange and Miethke [1933]. Table II gives some results.

Table II.

Sample	Net oxalate shift ml. *N*/10 per 100 ml. milk	Inorganic P as ml. *N*/10 H_3PO_4 per 100 ml.		
		Total	Soluble	Insoluble
A	8·8	20·0	11·7	8·3
B	8·8	19·9	10·9	9·0
C	12·2	21·7	8·7	13·0

The agreement between the values in the first and last columns is quite close. For strict comparison the former still require to be reduced by about 0·3 ml. *N*/10—approximately the degree of alkalinity to phenolphthalein developed in 100 ml. 3 % calcium caseinogenate on addition of the amount of oxalate here used—a correction which does not materially affect the results. These show that the greater part of the colloidal phosphate, if not all, reacts to oxalate as tricalcium phosphate.

Preliminary experiments on caseinogen-calcium phosphate complexes.

These experiments were made primarily in order to see how far the effects of oxalate on milk could be reproduced on synthetic materials, and they are somewhat qualitative. More detailed examination of the phosphatic complexes is reserved for the next section. It was found that these complexes whether obtained by the action of phosphoric acid on alkaline calcium caseinogenate at about 0° or formed in similar conditions by the successive slow addition of calcium chloride and disodium phosphate solutions to one of nearly neutral caseinogenate (a process which in the absence of caseinogenate gives $CaHPO_4$), showed in all cases, in common with milk, the property of becoming much more alkaline on addition of oxalate, and thus appeared to contain some tricalcium phosphate.

The following results found for complexes prepared by the second method illustrate this point.

[1] The applicability of this oxalate method for the approximate determination of small amounts of tricalcium phosphate, and the correctness of these statements have been checked experimentally on solutions or suspensions of $CaHPO_4$ and $Ca_3(PO_4)_2$, including colloidal suspensions of the latter in gelatin. The last react with oxalate somewhat slowly.

Table III.

	Total P added as ml. $M/10$ H_3PO_4 per 100 ml.	Total oxalate shift of complex ml. $M/10$ per 100 ml.	Oxalate shift of papain filtrate ml. $M/10$ per 100 ml.	Net oxalate shift ml. $M/10$ per 100 ml.
Complex A	11·1	8·2	1·4	6·8
„ B	10·0	7·4	1·5	5·7

An approximate titrimetric estimation of the soluble phosphate in the papain[1] filtrates gave 4·1 ml. $M/10$ and 4·6 ml. $M/10$ per 100 ml. respectively for A and B, so that the $CaHPO_4$ which might have been expected from the method of preparation is apparently replaced in these experiments by almost equal amounts of tricalcium and of soluble phosphates.

No doubt the reaction of the solution changed during the formation of these phosphates, and would need to have been followed to allow more quantitative interpretation of the results, but the conclusion that tricalcium rather than dicalcium phosphate is formed can hardly be avoided. The following experiment offers further confirmation of this view.

A solution of $M/5$ disodium phosphate of p_H 8·47 was added in successive small amounts to a 3 % calcium caseinogenate solution of about the same p_H, *viz.* 8·4, and the hydrogen ion concentration of the mixture was determined after each addition, using the glass electrode. If dicalcium phosphate is formed from the interaction of the two solutions as the inorganic colloid associates with the caseinogenate, the mixture should not differ greatly in p_H from its constituents. If however tricalcium phosphate is formed, from what may be regarded as the elements of $CaHPO_4$, the resulting mixture should become more acid, particularly in the earlier stages. The experimental results, shown diagrammatically in Fig. 1 are in complete accordance with the latter view. To demonstrate that the changes in p_H which occurred on addition of the phosphate to the caseinogenate were not due merely to partial interchange of bases in the latter, *etc.*, a similar series of potentiometric titrations was made on part of the same calcium caseinogenate solution treated with neutral $M/5$ potassium oxalate. These results, shown also in Fig. 1, are entirely different from those with phosphate.

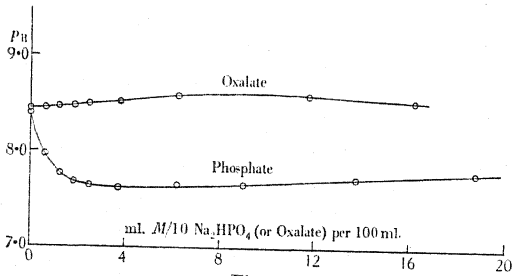

Fig. 1.

Distribution of inorganic phosphorus in caseinogen-calcium phosphate complex.

Porcher and Chevallier [1923] concluded that the colloidal calcium phosphate was the tertiary salt, but Porcher later [1930] formed the opinion, from experiments on the artificial preparation of caseinogen-calcium phosphate complexes,

[1] These solutions contained too little soluble calcium to allow the use of rennet in preparing the serum and papain was employed instead, as by Kometiani [1931].

that the inorganic colloid was neither entirely secondary nor tertiary but a mixture of the two. The complexes were prepared by the addition of sufficient phosphoric acid to decidedly alkaline calcium caseinogenate to bring the whole to about p_H 7·0. On the assumption that the calcium belonging properly to the protein is approximately equal in the complex to that found in pure calcium caseinogenate of the same p_H, he found that 1 g. mol. H_3PO_4 was required in order to neutralise approximately 1·25 g. mol. of excess CaO, a result which corresponded to the formation of di- and tri-calcium phosphates in almost equal proportions. Porcher recognised that a small amount of monocalcium phosphate was formed during the neutralisation, and that dicalcium phosphate is itself slightly soluble, but does not seem to have considered quantitatively the bearing of these facts on the type of colloidal phosphate formed at the same time. It is clear that they may influence the interpretation of the titration results considerably. For, if soluble phosphate is present at a p_H of about 7, it will consist of a mixture of nearly equal amounts of monocalcium and dicalcium phosphates, for which CaO/H_3PO_4 in g. mols. = 0·75. It is only necessary for one-fourth of the total phosphate to be present in this form in order to account for an experimentally found ratio CaO/H_3PO_4 in g. mols. = 1·25, on the basis of the other three-quarters being entirely tertiary.

It was decided therefore to repeat the preparation of these complexes, determining at the same time the proportion of the added phosphate which remained in solution. For this purpose a calcium caseinogenate solution containing about 5 % of the protein and having p_H about 7·0 was prepared by trituration of "Hammarsten casein" with lime water, allowed to stand overnight in an ice-chest and then filtered through hardened paper. To 50 ml. of the filtrate were added 30–40 ml. saturated lime water, and then $M/20$ phosphoric acid was slowly run with constant rapid mechanical stirring into the ice-cooled mixture until it had attained approximately its original p_H of 7·0. After some hours the hydrogen ion concentrations of the phosphate complex and of the original filtered calcium caseinogenate (similarly diluted with water) were determined with the glass electrode. One or two further determinations were also made on the latter solution after the addition of small quantities of lime water, so as to be in a position to allow accurately for the calcium directly associated with caseinogen in the complex, in case the p_H values of complex and original caseinogenate should differ slightly.

The total inorganic calcium and phosphorus contents of the complex were, of course, known from the amounts of lime water and phosphoric acid used in its preparation, but the determination of the soluble phosphate required the removal of the caseinogen-calcium phosphate complex from solution without disturbing the equilibrium of the soluble constituents. Rennet was unsuitable for this purpose as the soluble calcium content was too low and could not be increased, *e.g.* by addition of a soluble calcium salt, without danger of upsetting the equilibrium of the system. The enzyme papain, used successfully by Kometiani [1931] for preparing the serum of heated milk, proved unsatisfactory in the present instance, since the filtrate obtained could not be rendered sufficiently free from nitrogenous matter by trichloroacetic acid and gave cloudy solutions with the Briggs molybdate reagent. Ultrafiltration was finally adopted for the preparation of the serum and the phosphate determined colorimetrically as already mentioned. The calcium associated with phosphate in solution was calculated from the latter for the particular p_H of the complex, using Sørensen's value for the phosphate buffers [*cf. e.g.* Clark, 1928]. The results are shown in Table IV. The figures in the last column entitled "corrected oxalate shift"

Table IV.

% protein	p_H	Inorganic P as ml. $M/10$ H_3PO_4 per 100 ml. Total	Soluble	Insoluble	Inorganic Ca as ml. $M/10$ $Ca(OH)_2$ per 100 ml. Total	Soluble (calc.)	Insoluble	Ratio ml. $M/10$ $Ca(OH)_2$ / ml. $M/10$ H_3PO_4 Total	Insoluble	Corrected oxalate shift ml. $N/10$ per 100 ml.
2·37	6·97	7·40	—	—	9·15	—	—	1·24	—	5·0
2·05	6·91	5·90	1·48	4·42	7·29	1·15	6·14	1·24	1·39	4·0
2·11	6·87	6·60	1·28	5·32	8·51	0·99	7·52	1·28	1·41	4·5

represent the alkalinity developed in the complex on addition of the usual amount of oxalate, less an allowance of 0·5 ml. N to cover the sum of the alkalinities developed in 2 % calcium caseinogenate and the ultrafiltrate from the complex on similar treatment.

The ratio of total inorganic calcium to phosphorus is seen to agree closely with that found by Porcher, but, as expected, allowance for that fraction of these elements present in solution raises the ratio of the remainder which is available for the formation of colloidal phosphate to about 1·40. As the corresponding ratios for dicalcium and tricalcium phosphates are 1·0 and 1·5 respectively, it is clear that this result requires the bulk of the colloidal phosphate to be tertiary but does not exclude however the possibility that some dicalcium phosphate is also present. The values for the shift in reaction of the complex towards alkalinity on addition of oxalate, shown in the last column, are seen to be slightly lower than those for insoluble or colloidal phosphate and thus also support this possibility.

In spite of the suggestion from these results that the colloidal phosphates may be partly secondary, there are other considerations which favour the simpler view that the colloid is entirely tertiary and that the slight divergencies in these experimental results from what might have been expected in such circumstances are due to interfering causes. For instance, it has been assumed that the caseinogen-calcium phosphate complex and the corresponding caseinogenate free from colloidal phosphate will possess the same p_H values in solution. While some such assumption is necessary to prevent the problem becoming indeterminate, it is probably not correct to expect that the two corresponding substances will really have identical p_H values, although they will probably not differ greatly in this respect. Differences in the degree of hydrolysis, *etc.*, which occur, for instance, between the alkali and alkaline earth caseinogenates, are likely to operate here also. Thus, results which make a caseinogen-calcium phosphate complex appear to consist, somewhat improbably, of calcium caseinogenate associated with a mixture of phosphates of which some 80 % is tertiary and 20 % secondary may more properly be susceptible of the simpler interpretation that the complex consists of caseinogenate and tricalcium phosphate only, the union of which is accompanied by a slight change in reaction. Evidence on the point was sought by means of potentiometric titration.

Potentiometric titration of calcium caseinogenate and caseinogen-calcium phosphate complex.

On the assumption that the amount of calcium associated directly with caseinogen in the complex is identical with that present in calcium caseinogenate of the same p_H, the titration curves of the two substances (freed of course from soluble phosphate) given with calcium hydroxide will be exactly similar. If

however this assumption is not strictly correct, as is suggested, the titration curves should differ somewhat. Table V gives the results of some experiments, one of which is also shown graphically in Fig. 2. Solutions of calcium caseinogenate and of various phosphatic complexes prepared from them were made and matured, as previously described, and then dialysed side by side in cellophane sausage skin dialysers for (mostly) 18 hours to remove soluble phosphate from the complex. A little mustard oil was used as a preservative. The solutions which contained some 2–3 % protein were then titrated potentiometrically with lime water, using the glass electrode and allowing an interval of 10 minutes for attainment of equilibrium between each addition of alkali and the succeeding measurement.

Table V.

Exp. 1

Dialysis (18 hrs.)

Complex		Caseinogenate	
ml. $N/10$ $Ca(OH)_2$ per g.	p_H	ml. $N/10$ $Ca(OH)_2$ per g.	p_H
0·0	6·94	0·0	6·85
1·10	7·82	1·12	7·68
2·20	8·73	2·24	8·73
3·30	9·34	3·36	9·26
4·40	9·71	4·47	9·57

Exp. 2

Dialysis (18 hrs.)

Complex		Caseinogenate	
ml. $N/10$ $Ca(OH)_2$ per g.	p_H	ml. $N/10$ $Ca(OH)_2$ per g.	p_H
0·0	7·10	0·0	6·82
0·78	7·71	0·80	7·63
1·56	8·54	1·60	8·66
2·34	9·17	2·41	9·11
3·12	9·45	3·21	9·36

Exp. 3

Dialysis (18 hrs.)

Complex		Caseinogenate	
ml. $N/10$ $Ca(OH)_2$ per g.	p_H	ml. $N/10$ $Ca(OH)_2$ per g.	p_H
0·0	7·10	0·0	6·79
0·69	7·55	0·68	7·41
1·38	8·13	1·36	8·27
2·07	8·76	2·04	8·88
2·76	9·30	2·72	9·16
4·14	9·71	4·08	9·58

Dialysis (66 hrs.)

Complex		Caseinogenate	
ml. $N/10$ $Ca(OH)_2$ per g.	p_H	ml. $N/10$ $Ca(OH)_2$ per g.	p_H
0·0	7·19	0·0	6·79
0·73	7·82	0·72	7·28
1·46	8·67	1·44	8·11
2·18	9·24	2·17	8·69
2·91	9·48	2·89	9·09
4·36	9·90	4·33	9·57

All the results show that the two titration curves are not identical in shape, the buffer value of the complex being somewhat greater than that of the caseinogenate in the earlier stages of the titration, *e.g.* from about p_H 7·0 to p_H 8·5, and lower thereafter. The type of difference found in the earlier stages

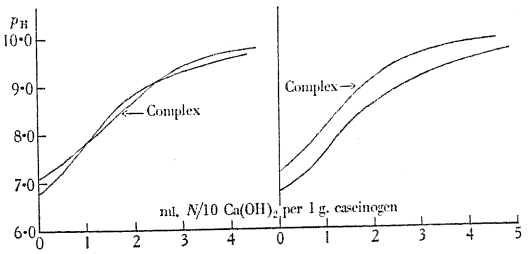

Fig. 2.

was somewhat unexpected and was perhaps partly due to the presence of small amounts of soluble phosphate which had escaped diffusion, for it was less evident on longer dialysis (*cf.* curves for 18 and 66 hours' dialysis, Fig. 2). Conclusions

drawn from the results of prolonged dialysis must be accepted with some reserve however since it is difficult completely to eliminate bacterial changes which may alter the shape of the titration curves.

It is clear from the present results however that the titration curves of the caseinogenate and its calcium phosphate complex are not identical, and that is the main point, for it indicates that the quantity of calcium associated with the protein in the complex may differ somewhat from that attached to the isolated protein at the same p_H. This finding favours then the more natural view that the complex consists of calcium caseinogenate and tricalcium phosphate, uniting probably with some slight change in reaction, rather than that which, on a strict interpretation of the synthetic experiments, would make it appear to consist of calcium caseinogenate united with a perhaps variable mixture of phosphate (chiefly tertiary) and possessing a p_H identical with that of the parent caseinogenate.

It is hoped to return to a more quantitative consideration of these and similar titration curves on a future occasion. It may be remarked here however that the differences between them provide additional evidence of the existence of a chemical union between calcium caseinogenate and calcium phosphate.

SUMMARY.

1. Further experiments on the effect of colloidal calcium phosphate (and carbonate) on the formaldehyde titration of calcium caseinogenate are described, and are interpreted as showing the existence of some type of chemical union between the calcium caseinogenate and the colloidal calcium phosphate of milk.

2. The effect of oxalate on the titratable acidity of milk and caseinogen-calcium phosphate complexes is examined. It appears to indicate that the inorganic colloid constituent is mainly if not entirely tertiary phosphate, a conclusion supported also by the results of experiments on the preparation and electrometric titration of the complexes.

3. The results, taken together, suggest that the caseinogen-calcium phosphate of milk has the nature of a double salt of calcium caseinogenate and tricalcium phosphate.

REFERENCES.

Clark (1928). Determination of hydrogen ions. (Baillière, Tindall and Cox, London.)
De Toni (1921). *Kolloid. Z.* **28**, 145.
Grimmer (1926). Lehrbuch der Chemie u. Physiologie d. Milch, p. 71 (2nd ed. Berlin).
György (1923). *Biochem. Z.* **142**, 1.
Hammarsten (1879). *Bied. Centr.* 147.
Hoffman and Gortner (1923). *J. Physical Chem.* **29**, 769.
Kometiani (1931). *Milchwirtschaftl. Forsch.* **12**, 433.
Lange and Miethke (1933). *Milchwirtschaftl. Forsch.* **14**, 195.
Lehmann (1894). *Pflüger's Archiv.* **56**, 558.
Lindet (1912). *Ann. Inst. Nat. Agronom.* (2), **11**, fasc. 2.
Palmer (1924). *Ind. Eng. Chem.* **16**, 631.
Porcher (1930). Le lait au point de vue colloidal. Lyon.
—— and Chevallier (1923). *Le Lait*, **3**, 188.
Pyne (1932). *Biochem. J.* **26**, 1006.
—— and Ryan (1932). *Sci. Proc. Roy. Dub. Soc.* **20** (N.S.), 471.
Söldner (1888). *D. Landwirtschaftl. Versuchs Stat.* **35**, 351.
Wright (1928). *J. Agric. Sci.* **18**, 478.
Van Slyke and Bosworth (1914, 1). *New York Agric. Exp. Sta. Tech. Bull.* No. 37.
—— —— (1914, 2). *New York Agric. Exp. Sta. Tech. Bull.* No. 39.

CXXXV. POTENTIOMETRIC STUDY OF PHOTO-FLAVIN.

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It has previously been shown that several members of the recently described flavin or lyochrome series of pigments exhibit the properties characteristic of reversible oxidation-reduction systems [Stern, 1934, 1].

Photolysis of the flavin molecule in alkaline solution yields a pigment of smaller molecular weight which retains the colour, fluorescence and reversibility of oxidation-reduction of the parent compound and in addition is soluble both in chloroform and in water, whereas the native flavins are insoluble in chloroform [Warburg and Christian, 1932]. Following the discovery by Warburg and Christian of this peculiar reaction in the case of yeast-flavin which these authors designate "gelbes Oxydationsferment," the same process was found to occur in all other flavins which were tested, *i.e.* ovoflavin and lactoflavin (vitamin B_2) [Kuhn and Wagner-Jauregg, 1933; Kuhn, György and Wagner-Jauregg, 1933; Ellinger and Koschara, 1933], hepatoflavin, uroflavin and maltoflavin [Stern, 1933; 1934, 1].

From horse-liver fractions which had been obtained by adsorption and elution as described in the experimental part of this paper photo-hepatoflavin was obtained by the procedure devised by Warburg and Christian for yeast; 9 kg. of liver yielded thus between 6 and 10 mg. of recrystallised product. Not only were the colour, the shape and arrangement of the micro-crystals, and the other general properties of this compound obviously the same as those given by Warburg and Christian for their photo-product from yeast-flavin, but the melting-point and the spectroscopic data of these two substances were within certain limits the same. A similar agreement exists between photo-hepatoflavin and the "lumi"-lactoflavin of Kuhn, Rudy and Wagner-Jauregg [1933]. It is remarkable that the absorption spectra of the non-irradiated ovoflavin and lactoflavin are much the same as those of the different photo-flavins [Kuhn, György and Wagner-Jauregg, 1933]. It follows therefrom that the sugar-like grouping $C_4H_8O_4$ which is detached from the coloured part of the flavin molecule by the photo-reaction does not affect the light-absorption of the complex molecule. Similar observations have been reported in the study of nucleosides [Holiday, 1930].

A more detailed account of spectrographic work on hepatoflavin and photo-hepatoflavin done jointly with Dr E. R. Holiday will be presented in a separate publication. It will suffice therefore to tabulate here the molecular extinction coefficients of photo-hepatoflavin in 0·01 *N* NaOH as compared with those of photo-yeast-flavin, lumi-lactoflavin and ovoflavin, as given by Warburg and Kuhn respectively.

Table I.

Photo-hepatoflavin sublimed, in 0·01 *N* NaOH		Photo-yeast-flavin (chloroform)		Lumi-lactoflavin (chloroform)		Ovoflavin (water)	
λ	x	λ	x	λ	x	λ	x
445 $m\mu$	$1{\cdot}19.10^4$	452 $m\mu$	$2{\cdot}58.10^4$	445 $m\mu$	$2{\cdot}6.10^4$	446 $m\mu$	$2{\cdot}11.10^4$
352	$1{\cdot}49.10^4$	350	$1{\cdot}94.10^4$	360	$1{\cdot}7.10^4$	366	$1{\cdot}75.10^4$
267	$5{\cdot}89.10^4$	270	$6{\cdot}16.10^4$	270	$7{\cdot}5.10^4$	267	$8{\cdot}5\ .10^4$

$$\left(x = \text{mol. extinction coeff.} = \frac{2{\cdot}30}{c.d} \log \frac{I_0}{I}\right).$$

With sufficient amounts of the crystallised photo-hepatoflavin in hand it seemed to be of interest to undertake a study of its behaviour as an oxidation-reduction system by means of potentiometric analysis. This was done to collect data which might be useful for a comparative study of the potentials of various flavins, but especially to gain some insight into the structure of the special grouping of the molecule which is responsible for its function as a redox-system *in vitro* and probably as an intermediary redox catalyst (carrier) *in vivo*. The fact that the photo-flavins are artefacts and represent only a part of the original flavin molecule is of course not to be overlooked. But since the photo-flavins retain so many features of the native compounds and especially since they still incorporate the arrangement of atoms responsible for both colour and reversible oxidation-reduction, it may be hoped that the information obtained by this study will have a certain bearing on the problem of the flavins in general. Incidentally it was found that the system under investigation is one capable of semiquinone formation and is therefore accessible to a theoretical treatment of the experimental data along the lines recently developed by L. Michaelis and his colleagues. Kuhn and Wagner-Jauregg [1934] observed in qualitative experiments that, whereas the colour change on reduction of the flavins in neutral or alkaline solution is from yellow to colourless, in strongly acid solution it is from yellow over an intermediary red stage to colourless. They advanced the hypothesis that the red colour belongs to an organic radical of the character of a semiquinone. Certain analogies with pyocyanine, to which Kuhn referred in a recent lecture [1934], were however invalidated by the use of an erroneous value of the pyocyanine potential [Stern, 1934, 2]. Moreover there was no evidence as to the nature of the red compound.

It will be shown that the oxidation or reduction of photo-flavin in acid solution is performed in two steps each involving the exchange of one electron, a complete separation of the two processes only being effected in very acid solutions. The intermediary stage may be depicted as a semiquinone, *i.e.* a partly reduced and partly oxidised molecule of remarkable stability in view of its character.

The author was fortunate enough to secure a small sample of crystalline photo-yeast-flavin prepared by Prof. Otto Warburg and Dr Walter Christian. This sample was presented to Prof. E. C. Dodds in July 1933 and stored since then at 0°. My best thanks are due to Prof. Warburg and to Prof. Dodds for their kindness in placing this valuable substance at my disposal.

Comparative measurements at three different p_H values showed that in addition to the above-mentioned properties the potentials of the photo-flavins are also in close agreement. The conclusion seems therefore to be warranted that the photo-flavin from hepatoflavin is identical with that from yeast-flavin and most probably with the photo-products of the other flavins.

When this work was already well advanced a publication by Bierich and Lang [1934] dealing with the potentials of a yellow pigment from mammalian tissues came to our knowledge. In this paper the data given by Bierich *et al.* [1933] in a preliminary communication were somewhat extended. The authors do not describe in detail the preparation or the properties of their pigment. They state that only very small quantities of the impure pigment in solution were at their disposal. There is no mention of the spectroscopic properties. No single titration curve is reproduced and the authors refer to their curves as being "typically S-shaped" and indicating an electron number $n=2$. To corroborate this statement the index potential is given as 14·6 and 14·7 mv. The curve relating the normal potentials at varying hydrogen ion concentrations from p_H 1 to 11·4 exhibits values which differ from our values by as much as 30 mv. With the exception of one experiment all titrations performed by Bierich and co-workers were oxidative ones with ferricyanide as titrant. We find that titrations with ferricyanide at low p_H yield no curves capable of interpretation owing to the formation of a blue-green complex in acid solutions, which seems to be of irreversible nature. The authors failed to observe the red intermediate and the steepening of the titration curves in the acid range where the semiquinone is formed. At my request Prof. R. Bierich provided me with a more detailed description of the preparation of his pigment. Following this a small amount of pigment in solution was obtained from 500 g. liver. The spectrographic examination, kindly carried out by Dr E. R. Holiday, revealed an absorption spectrum very similar to that of the photo-flavins. The pigment could be reversibly reduced and oxidised and the red intermediate could be observed in mineral acid solution. It appears therefore that Bierich has been working with impure photo-flavin and that the discrepancies between his results and ours are to be ascribed to the impurity of his preparation and, as far as the acid range is concerned, to the different experimental procedures.

Experimental.

Preparation of photo-hepatoflavin.

4·5 kg. of fresh horse-liver are freed from fat and connective tissue and are finely minced in an electrical mincer. The liver mash is stirred into 6 l. of boiling tap-water and boiling is maintained for 10 minutes. After cooling to about 40° the decoction is pressed by hand through press-cloth. To the slightly turbid yellow-brown extract about 50 ml. conc. HCl and 250 g. fuller's earth are added and adsorption carried out by mechanical shaking for one hour. The remaining solution is separated from the adsorbate by means of a centrifugal filter-drum lined with press-cloth. After washing with at least 4 l. distilled water the hepatoflavin is eluted from the fuller's earth by shaking it twice with 1·5 l. elution mixture (1 l. distilled water *plus* 250 ml. methyl alcohol *plus* 250 ml. pyridine) for 1 hour. The eluate is obtained preferably by suction through a Büchner funnel in which a thin layer of kieselguhr is spread over two ordinary paper filters. The clear solutions show a deep yellow colour and a strong green fluorescence even in ordinary daylight. They are jointly concentrated *in vacuo* at approximately 40° to about 500 ml. The remaining pyridine is removed by treatment with about 1 l. ether in three portions. The watery layer is brought to an NaOH content of 0·5 *N* by the addition of 10*N* NaOH. A colourless precipitate is formed which is removed by filtration. The dark yellow solution is irradiated for at least 5 hours from a 100 c.p. metal filament lamp at 15 cm. distance. The temperature is kept below 20° by spreading the solution in a glass

dish which is immersed in a larger glass basin through which tap-water flows rapidly. The light-source is installed beneath the cooling bath. During irradiation the solution is mixed by stirring with nitrogen or air. After photolysis is completed, which may be ascertained by carrying out a chloroform test on a small sample, the solution is acidified with HCl to Congo red, whilst carefully cooled with ice-water. It is then extracted three times with three lots of about 300 ml. benzene, which removes practically no pigment. The photo-flavin is extracted by frequent extractions with small amounts of chloroform until the chloroform layers are only faintly coloured. Though the watery layer may still show some yellow colour, owing probably to non-flavin pigments, it is discarded and the combined chloroform extracts (about 1500 ml.) are filtered through a filter moistened with chloroform or are centrifuged to remove residual water and also colourless flocks. The chloroform solution is then concentrated *in vacuo* to about 300 ml. and the photo-flavin is extracted with 100 ml. 0·1 *N* NaOH. The yellow alkaline solution is acidified with HCl to Congo red and the photo-product is extracted by shaking with small amounts of chloroform. The exchange between water and chloroform phases is repeated at least three or four times. The last chloroform extract is filtered and the chloroform completely removed *in vacuo* or in a stream of cold air. The thin yellow-brown film is dissolved in dilute alkali (about 20 ml.), filtered and just acidified with HCl. Usually a faint turbidity appears. On slow concentration of the solution *in vacuo* over sulphuric acid, photo-flavin crystals gradually appear. When the volume of the solution is about one-fourth of the original the crystals are filtered off or separated from the mother-liquor by centrifuging. After washing with cold water in which they are only sparingly and slowly soluble the crystals are dried *in vacuo* over sulphuric acid. The yellow-brown needles melt with decomposition about 340° (uncorrected). They may be sublimed in a high vacuum at 230° without decomposition.

Arrangement and methods of potentiometric measurements.

In general the technique developed by Clark and by Michaelis for measuring oxidation-reduction potentials was employed. A Tinsley research potentiometer was used, the scale of which reads to 1 mv. and allows a fair estimation of 0·1 mv. Since the internal resistance of the instrument is comparatively low (40 ohms) a resistance of 1500 ohms was connected in series with the reference electrode and titration electrodes. As indicator a Tinsley deflection galvanometer of the lamp and scale type was used. The sensitivity was given by the maker as 100 scale divisions per milliamp. The period was one second. As normal element a cadmium cell was used that had been calibrated by the Physikalisch-Technische Reichsanstalt, Berlin (1019·3 mv.). The actual titration outfit consisting of a saturated calomel electrode, and the titration vessel was placed in a spacious air-thermostat that was kept at $30{\cdot}0 \pm 0{\cdot}5°$. The volume of the titration vessel was 30 ml., the volume of liquid necessary to cover the electrodes completely was 10 ml. As experimental electrodes one gold-plated and one bare platinum electrode were used. The titrant was delivered from a micro-burette holding 1 ml. and graduated in 0·01 ml. The calomel electrode was frequently standardised by means of the hydrogen electrode in standard acetate and gave almost invariably a potential difference against the latter of 515 to 515·5 mv. For the measurement of $[H^+]$ three methods were employed alternately and sometimes two of them were applied to the same solution: (*a*) measurement of p_H with the U-hydrogen electrode of Michaelis; (*b*) reduction of the buffered pigment solution by colloidal palladium and hydrogen and estimation of $[H^+]$ from the potential values of the experimental electrodes,

previous to oxidative titration with ferricyanide [Michaelis and Eagle, 1930]. It was found unnecessary to provide for a separate platinum electrode covered with platinum black, since after a sufficient time of hydrogenation both the gold-plated and the bare platinum electrodes gave identical and true potential values as controlled with method (*c*). This may be due either to incomplete smoothness of the electrode surfaces or to the presence of the colloidal palladium in the system which might form a small deposit on the electrodes and make them act as palladium electrodes which are known to be perfect hydrogen electrodes[1]. (*c*) The standard method, however, employed in the present study was the measurement with the glass electrode arrangement as developed by Greville and Maclagan [1931] at this Institute. I am indebted to Mr M. E. H. Fitzgerald for these measurements.

The hydrogen and nitrogen used for the titration experiments were taken from cylinders containing commercial gases of 99·9 % purity. Complete purification was achieved according to Michaelis [1931, 1]. A 5 m. copper tube was used to lead the purified gases into the titration vessel. This ensured a rapid cooling of the gas heated to approximately 500° in the furnace. Even in the most negative potential ranges no drift of the potentials towards the positive range which would indicate the presence of traces of oxygen could be observed.

The electrometric titrations for ascertaining the position of the dissociation constants were performed in the same apparatus with the exception of the electrodes used. Two platinum wires hammered flat at the end and covered with a platinum black deposit of medium thickness were submerged in 12 ml. solution containing 2 mg. (10^{-5} mol.) recrystallised photo-hepatoflavin *plus* 0·1 ml. palladium sol. The titrant was 0·01 *N* NaOH. Throughout the experiment the pyrex titration vessel was flushed with a stream of pure hydrogen at 30°. Whereas the dissociation constant of the reduced form of the pigment could be determined in this way, an attempt to apply this technique (with the omission of the palladium sol) to the oxidised form failed owing to a complete disagreement of the electrodes and weakly maintained potentials. This may have been caused by the competition for the electrode surface between the oxidation-reduction system and the hydrogen ions present in the solution.

Results.

Titration experiments on photo-hepatoflavin.

To permit an adequate description of this system 26 titration experiments were performed at varying hydrogen ion concentrations, the limits being p_H −0·2 and 12·4. The molar concentration of the photo-flavin solutions was of the order 10^{-3} to 10^{-4}. Except in the very acid range where HCl and H_2SO_4 were used, buffers of sufficient strength (0·5 to 0·1 *M*) were employed to ensure a constant $[H^+]$ during the experiment. The p_H measurements with the glass electrode were usually made after the titration was finished.

The first series of titrations dealt with the behaviour of the system in the neutral and alkaline ranges. Here the colour change is from pure yellow to colourless on reduction and *vice versa* on oxidation of the leuco-compound. In this range the system behaves exactly like one of the well-known organic redox-indicators, *e.g.* methylene blue. The electrodes adjust themselves almost instantaneously to the potential changes brought about by the variation of the oxidant/reductant ratio on addition of titrant, so far as the range from 10 to 90 %

[1] The possibility of determining p_H in this way was discovered by Biilman [(1927) *Z. physikal. Chem.* **130**, 566]. I am indebted to Prof. L. Michaelis for pointing this out.

reduction is concerned. Here the agreement between the gilded and bare platinum electrodes is perfect within 0·5 and many times within 0·1 mv. No perceptible potential drift is observed up to 30 minutes. Current of the strength limited by the resistance of the chain may be drawn from the electrodes for several seconds without upsetting the potential readings or causing polarisation. Outside the range of sufficient poising the potentials are established only after some time—10–40 minutes—has elapsed since the addition of the titrant. In this range the agreement between the different electrodes is usually less satisfactory and varies between 1 and 10 mv. It may be remarked that the same applies to the best reversible systems known. The slope of the titration curves in the range from p_H 6 to 12·4 is that of an ordinary two electron system with an index potential in the neighbourhood of 14 mv., within the limits of experimental inaccuracies and the error involved in the graphical interpolation for the determination of the mid-point of the curves. The latter is almost unavoidable in view of the fact that even in the case of the photo-flavin where the end of the titration is indicated by a pronounced jump of potential the selection of the actual end-point invariably introduces a certain arbitrary element[1].

In the indicated p_H range the experimental method exclusively used was that of Michaelis and Eagle [1930], consisting in the reduction of the system by means of hydrogen activated by Paal's colloidal palladium and by reoxidising the leuco-compound with ferricyanide. The ferricyanide used was of analytical purity and the strength of the solution was determined in two instances by iodimetric titration. The solvent was de-aerated before addition of the accurately weighed oxidant by a stream of pure nitrogen, and the solution as well as the amount transferred to the micro-burette were protected against oxygen by a thick layer of liquid paraffin.

The curves thus obtained between p_H 7·4 and 12·4 have been collected in Fig. 1 after transformation of the amounts of oxidant used into the corresponding degree of oxidation.

The best proof of the strict reversibility of the system under investigation is perhaps to be seen in the fact that several of the titration curves shown in this and also in the following diagram were obtained with the same specimen of photo-flavin solution, the p_H of which was adjusted to another value under electrometric control after each experiment was finished.

The second series of titrations deals with the phenomena in acid solutions. When attempts were made to apply the technique outlined above, which had given such satisfactory results in the neutral and alkaline ranges, to solutions of photo-flavin of $p_H < 6$ unexpected difficulties were met. At p_H 5·6 it was still possible to obtain an S-shaped curve as shown in Fig. 2, but the slope of the curve was much steeper in the first half of the experiment than in the second half. In the range below p_H 5 we did not succeed in obtaining S-shaped curves by means of the oxidative technique. With ferricyanide as oxidant non-characteristic curves were recorded. The potentials showed a rapid drift towards the positive side especially as the titration proceeded. No definite end-point corresponding to a definite jump of potential was reached in repeated attempts. With decreasing p_H values the appearance of a blue-green colour was noticed

[1] It should be remarked, however, that the conclusion concerning the nature of the process as a two-electron system has been derived from the slope of the titration curves, *i.e.* from the value of the index potential. The experimentally found position of the end-point fell invariably short of the stoichiometric equivalence point by as much as 50 and even 60 %. This holds true even on titration of photo-flavin purified by sublimation. This discrepancy is the object of further investigation.

as the titration proceeded. It became most pronounced when p_H 0 was approached. The colour persisted after admission of oxygen. On treating with chloroform no pigment could be extracted, which proves that the photo-flavin was actually transformed into a different compound. Sodium amalgam brought about a decoloration in mineral acid solution, which was apparently irreversible since no colour returned on vigorous shaking with air. It is planned to investigate this matter more thoroughly. At present the evidence is sufficient to warrant the statement that ferricyanide is obviously useless as a titrant in the acid range.

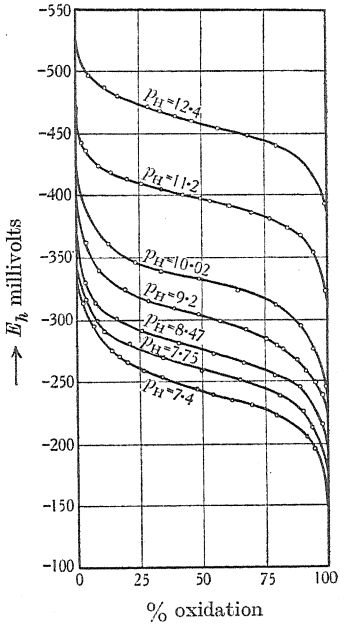


Fig. 1. Titration curves of photo-hepatoflavin at p_H 7·4 to p_H 12·4.

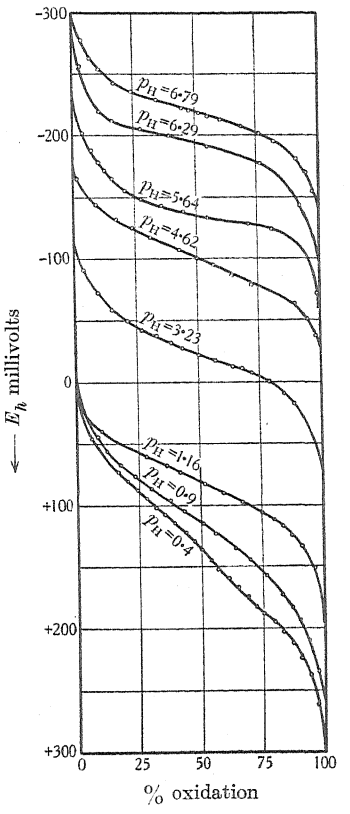


Fig. 2. Titration curves of photo-hepatoflavin at p_H 0·4 to p_H 6·79.

This is important in view of Bierich and Lang's [1934] assertion that ferricyanide may be used throughout the whole p_H range. Since attempts with dichromate and benzoquinone as oxidants at p_H 3·98 and 4·62 respectively did not yield quite satisfactory results either, it was suspected that the reduction procedure with palladium and hydrogen preceding the titrations might lead to different reduction products in acid and alkaline solutions respectively, more especially since it is known that hydrogenation of flavins may lead to the absorption of 1 and also of 3 mols of hydrogen per mol. flavin according to the experimental conditions chosen [Warburg and Christian, 1933; Kuhn, György and Wagner-Jauregg, 1933]. The following experiment was therefore devised and performed in co-operation with Mr G. D. Greville of this Institute. 0·7 mg. of a repeatedly

recrystallised sample of photo-hepatoflavin was dissolved in 3·5 ml. distilled water containing 0·2 ml. *N* NaOH. 1 ml. of this solution (corresponding to 0·2 mg. of photoflavin) was transferred to the main compartment of each of three Warburg vessels of the conical type. The volume of the solutions was made up to 2·0 ml. by addition of water and HCl (0·2 *N*) to adjust the p_H to 11·6, 5·17 and 1·74 respectively, as determined with the glass electrode after the experiment. KOH was used for absorption of CO_2 which might possibly be evolved. The side-bulbs contained 0·1 ml. colloidal palladium solution. An atmosphere of pure hydrogen was established by flushing the vessels attached to the manometers for 10 minutes with hydrogen which had passed the quartz copper furnace. After equilibration in the Warburg thermostat at 37° the palladium sol was tipped in from the side-bulb and the H_2 absorption followed for 125 minutes. A similar experiment was conducted with the omission of the photo-flavin. After subtraction of the hydrogen absorbed in this control experiment it was found that the photo-flavin had absorbed 14 μl. of hydrogen at p_H 11·6, 15 μl. at p_H 5·17, and 18·5 μl. at p_H 1·74. These hydrogen amounts

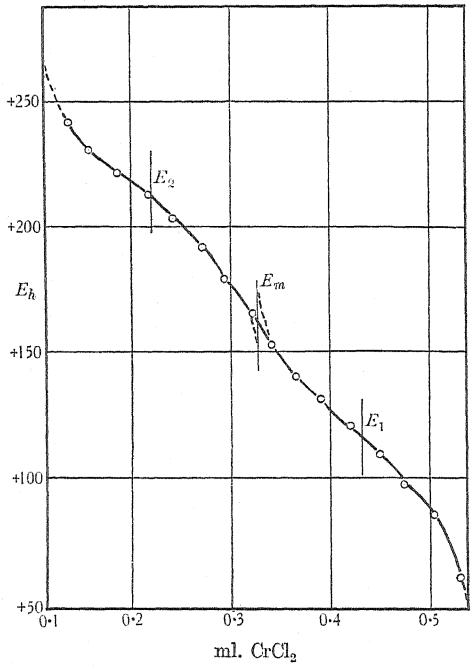


Fig. 3. Titration of photo-hepatoflavin with $CrCl_2$ at p_H – 0·2.

may probably be regarded as identical within the limits of error. Calculated per mg. photo-flavin an absorption of 70 to 92·5 μl. hydrogen was therefore observed as compared with 87 μl. absorption found by Warburg and Christian in weakly alkaline solution under similar conditions. The inference is that the difficulties encountered in the so-called oxidative titrations have their origin in the oxidative and not the reductive phase of the experiment.

Further work in the acid range was therefore conducted by exclusive adoption of the reductive titration method. Hydrosulphite, titanous chloride and

chromous chloride were used. Between p_H 5 and 0·4 titanous chloride proved to be very satisfactory. At still lower p_H the reductive power of this reagent was insufficient to bring about a definite jump in potential at the end of the titration owing to the approach to the hydrogen potential. Here chromous chloride with its extreme reductive power corresponding to a potential of hydrogen overvoltage was successfully used.

As will be seen from the composite diagram in Fig. 2 the slope of the titration curves becomes increasingly steeper with increasing [H+] below p_H 5. From p_H 4 to 1 the character of the curves is approximately that of a one electron system, the index potential varying between 20 and 25 mv. At $p_H < 1$ the steepness gradually increases until at p_H 0·4 the curve loses its uniform shape and two maxima appear. At this point evidently a separation in two steps of reduction is becoming manifest. On the titration of photo-flavin solutions below p_H 5 an intermediate orange tint is noticeable on reduction. With increasing [H+] this intermediate colour becomes gradually more and more pronounced until, as observed for the first time by Kuhn and Wagner-Jauregg [1934], a pure red appears midway between the yellow colour of the fully oxidised form

Table II.

Titration of crystalline photo-hepatoflavin in N HCl with $5 \cdot 10^{-3}$ M titanous chloride: Temp. 30°. p_H 0·4. E (potential difference between calomel and normal H_2 electrode) 0·2365 v.

(Calomel/standard acetate H_2 electrode 0·515 v.)

$TiCl_3$ ml.	Gold-plated Pt-electrode	Bright Pt-electrode	Mean	E_h		Colour	Remarks
					$E_{\frac{1}{4}}$		
					$E_{\frac{1}{2}}$		
					$E_{\frac{3}{4}}$		
0·00	+0·234	+0·2315	+0·2327	+0·4692		Lemon-yellow	Nitrogen
0·03	0·1755	0·1745	0·175	0·4115			
0·06	0·072	0·077	0·0745	0·311		Pure yellow	
0·095	0·022	0·0288	0·0254	0·2619		Yellow with pink shade	
0·125	−0·001	0·003	0·001	0·2375		Pink-yellow	
0·165	0·0135	−0·0102	−0·0118	0·2247			
0·195	0·0245	0·0225	0·0235	0·213		Red-yellow	
0·23	0·033	0·032	0·0325	0·204		Yellow-red	
0·265	0·0405	0·040	0·04025	0·1933	$E_{\frac{3}{4}}$		
0·30	0·0475	0·465	0·047	0·1895	0·187		
0·335	0·0545	0·054	0·0542	0·1823			
0·365	0·0618	0·061	0·0614	0·1751		Red	
0·40	0·069	0·0688	0·0689	0·1676		↓	
0·435	0·0768	0·076	0·0764	0·1601			
0·47	0·0855	0·085	0·0852	0·1513	$E_{\frac{1}{2}}$		
0·50	0·0925	0·092	0·0922	0·1443			
0·54	0·105	0·0995	0·100	0·1365	0·135	Red colour gradually fading	
0·575	0·108	0·107	0·1075	0·129		↓	
0·605	0·115	0·114	0·1145	0·122			
0·645	0·122	0·121	0·1215	0·115			
0·685	0·1285	0·1275	0·128	0·1085			
0·715	0·1359	0·1345	0·1352	0·1013	$E_{\frac{1}{4}}$		
0·75	0·1435	0·1425	0·143	0·0935	0·90		
0·785	0·1505	0·149	0·1497	0·0868		Pale pink	
0·82	0·1585	0·1565	0·1575	0·079			
0·855	0·167	0·1643	0·1657	0·0708			
0·96	0·1925	0·189	0·1905	0·046		Colourless	
	0·215	0·210				10 mins.	Palladium-hydrogen
	0·261	0·261				13	
	0·262	0·2625				35	
	0·2628	0·2628	0·2628			45	

p_H calculated from this value

Table III. *Summary of titration experiments performed on photo-hepatoflavin*[1].

Exp. No.	p_H	Buffer	Method	$E_{\frac{1}{4}}$	$E_0' = E_{\frac{1}{2}}$	$E_{\frac{3}{4}}$	E_i	Appar. n	0·03 log K $= E_{\frac{1}{4}} - E_i$	log K
29	−0·2	3 *N* H_2SO_4	Red. : $CrCl_2$	+0·106	+0·152	+0·202	0·048	0·6	+0·047	+1·6
26	+0·4	*N* HCl	Red. : $TiCl_3$	+0·090	+0·135	+0·187	0·048	0·6	+0·047	+1·6
25	0·9	Citrate-HCl	,,	+0·080	+0·113	+0·152	0·036	0·8	+0·03	+1
24	1·16	,,	,,	+0·058	+0·082	+0·105	0·0235	1·25	+0·006	+0·2
17	3·23	,,	Red. : $Na_2S_2O_4$	−0·044	−0·023	−0·003	0·0205	1·4	−0·006	−0·2
21	4·62	Acetate	,,	−0·124	−0·100	−0·074	0·025	1·15	+0·012	+0·4
2	5·64	Phosphate	Oxid. K_3FeCy_6	−0·149	−0·135	(−0·127)	0·014	2	−0·06	−2
6	6·29	,,	,,	−0·206	−0·194	−0·179	0·013	2·1	−0·06	−2
7	6·79	,,	,,	−0·234	−0·218	−0·202	0·016	1·75	−0·03	−1
1	7·41	,,	,,	−0·260	−0·243	−0·228	0·016	1·75	−0·03	−1
8	7·75	,,	,,	−0·275	−0·258	−0·242	0·0165	1·7	−0·03	−1
5	8·47	,,	,,	−0·293	−0·275	−0·257	0·018	1·55	−0·018	−0·6
12	9·2	Phosphate-NaOH	,,	−0·318	−0·303	−0·285	0·0165	1·7	−0·03	−1
3	10·02	,,	,,	−0·344	−0·332	−0·315	0·0145	1·95	−0·06	−2
23	11·2	,,	,,	−0·411	−0·397	−0·382	0·0145	1·95	−0·06	−2
10	12·4	,,	,,	−0·473	−0·458	−0·445	0·014	2	−0·06	−2

[1] Potential values referring to the normal H_2 electrode.

and the colourless leuco-compound. This occurs at $p_H > 1$–2. An almost complete separation of the two steps is reached at p_H −0·2 as shown in Fig. 3. Table II contains the protocol of the experiment at p_H 0·4, the corresponding curve of which is the lowest curve in Fig. 2. Maximum formation of the intermediate red colour thus occurs between the first quarter and the mid-point of the titration curve. Table III contains the data of the 16 experiments which correspond to the diagrams.

The foregoing observations and the fact that all the changes reported in colour and potential on titration of acid solutions of photo-flavin are fully reversible leave no doubt that the red intermediate is not a secondary reaction product but belongs to the group of quinhydrone-like substances which includes both the meriquinones and the semiquinones. According to Willstätter and Piccard [1908] meriquinones are molecular compounds composed of the oxidised and reduced forms of the same substance in which the ratio between the components need not necessarily be unity. The semiquinones on the other hand are molecules of partly reduced and partly oxidised character with an odd electron number and therefore of the nature of an organic radical, as depicted by Weitz [1928]. Though the existence of true meriquinones cannot be denied on the ground of mere theoretical considerations, potentiometric analysis by Michaelis and his colleagues and independently by Elema [1931; 1933] has in all cases tested led to the characterisation of such intermediates as semiquinones.

A survey of the experimental data of the present study leaves little doubt that the red intermediate in the photo-flavin reduction also belongs to the group of semiquinones. This is already evident from the fact that each of the two separate steps of the titration curve as obtained at p_H 0·4 or −0·2 is symmetrical in relation to the respective mid-point. The second criterion given by Michaelis, namely the independence of the value of the normal potentials of the two steps of the volume of the system, could not be tested owing to the scanty solubility of the photo-flavin in ranges of very low p_H and also to the small amounts of the compound at present available.

For the theory and mathematical analysis of the two-step oxidation with an intermediary semiquinone formation the reader is referred to the original

papers of Michaelis [1931, 2, 4; 1932] and also to the monograph by that author [1933]. For our purpose it may suffice to state that the values of the potentials recorded on titration of such a system are defined by the three equations:

$$E=E_m+\frac{RT}{2F}ln\frac{t}{r};\quad E=E_1+\frac{RT}{F}ln\frac{s}{r};\quad E=E_2+\frac{RT}{F}ln\frac{t}{s};$$

where E_m is the potential in the mid-point of the whole titration curve, E_1 the normal potential of the first and E_2 of the second step; t designates the concentration of fully oxidised (holoquinoid), r that of the fully reduced (benzenoid), and s that of the semiquinone stage. The distance of E_1 and of E_2 from E_m equals the actual distances $E_{\frac{1}{2}}-E_{\frac{1}{4}}$ and $E_{\frac{3}{4}}-E_{\frac{1}{2}}$ respectively as taken from the curve only if a complete separation of the single steps has been achieved. In the more frequent case of an overlapping of the single curves the distance of E_1 and E_2 from E_m may be estimated from the index potential by the aid of a table constructed by Michaelis [1933]. These values are given in the last but one column of Table III. The dismutation of the semiquinone into the holoquinoid and benzenoid forms proceeds according to the equation $s^2=krt$, where the mass action law constant k is called the formation constant of the semiquinone. Taking into consideration the dependence of degree of dissociation of the components of the system upon p_H, k also becomes a function of p_H and is designated then as the apparent or effective formation constant of the semiquinone. Its value is a measure of the probability and stability of semiquinone formation under a given set of experimental conditions. It is an empirical rule that semiquinone formation becomes manifest with increasing $[H^+]$. The last column of Table III contains the value of log k at varying p_H values.

Fig. 4 represents the essence of the present study. It gives in the fully drawn line the graphically determined mid-points of all titration curves plotted against p_H. This curve shows three inflections near p_H 0·4, 7·75 and 10. Between

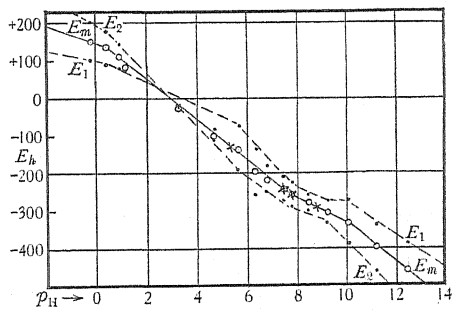


Fig. 4. Normal potentials of the holoquinoid, semiquinoid and benzenoid form of photo-flavin at varying $[H^+]$, referred to the normal hydrogen electrode at 30°. ⊙ Measurements on photo-hepatoflavin; × measurements on photo-yeast-flavin.

p_H 0·4 and 7·7 and also between 10 and 12·4 we find a rough approximation to a "60 mv. slope," whereas between −0·2 and +0·4, and also between 7·7 and 10 the slope is a "30 mv." one. This would mean that near p_H 0·4 and 10 are situated dissociation constants of the oxidised form and near p_H 7·7 one of the reduced form. The dotted lines represent the corresponding curves of E_1 and E_2 which intersect somewhere between p_H 1 and 4. Visible semiquinone formation of appreciable extent is obviously limited to the range of $p_H<2$.

Only here may the position of the normal potentials of the two steps be charted with confidence, whereas in the lower part of the E/p_H curve an uncertainty in the index potential of 1 mv. already greatly influences their position.

The following two features of the photo-flavin as a reversible redox system appear to be worthy of special mention: (1) the position of the normal potential at p_H 7 in a comparatively very negative region, namely at -227 mv., ranging on the scale of known systems between 1:5-anthraquinonedisulphonate (E_0' at p_H 7: -200 mv.) and β-anthraquinonesulphonate ($E_0' = -250$ mv.) [*cf.* Stern, 1934, 3]; (2) the remarkable stability of all the three possible forms of photo-flavin, *i.e.* of the holoquinoid, semiquinoid and benzenoid modifications.

The position of a dissociation constant of the fully reduced form near p_H 7·7 could be independently demonstrated by electrometric titration with NaOH in the manner described on p. 953.

Titration experiments on photo-yeast-flavin.

Three oxidative titrations on solutions of the crystalline photo-product of the yellow oxidation enzyme from yeast (Warburg) with palladium-hydrogen as reductant and ferricyanide as oxidant were performed at p_H 5·34, 7·4 and 8·73 respectively. The result of the experiment at p_H 7·4 is shown graphically in Fig. 5. The E_0' as taken from this graph is -244 mv. and is therefore identical

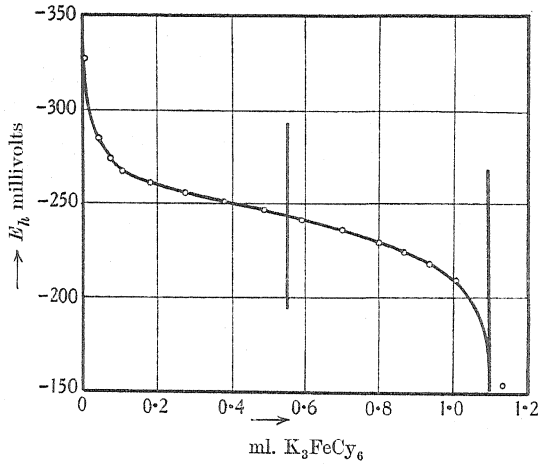


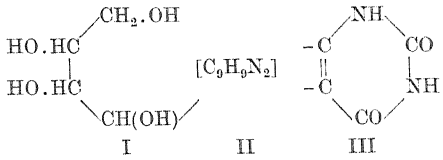
Fig. 5. Curve obtained on oxidative titration of the photo-product of the yellow oxidation enzyme (Warburg) at p_H 7·4. $E_0' = -0{\cdot}244$ mv.

with the corresponding value for photo-hepatoflavin at the same p_H (-243 mv.) within the limits of experimental error. The values obtained for the normal potentials at p_H 5·34 and at 8·73 are represented as crosses in the main diagram Fig. 4. It may be seen that these values are situated on the E_0'/p_H curve for the photo-hepatoflavin.

This fact is further and independent evidence that the two photo-flavins (and very probably all the known photo-flavins) are identical.

Discussion.

To the native flavin molecule (but without the colloidal bearer to which the flavin may be attached as a prosthetic group) the following tentative formula has been assigned by Kuhn, Rudy and Wagner-Jauregg [1933]:



I II III

In the course of the photo-reaction, formulated by these authors as

$$C_{17}H_{20}O_6N_4 - C_4H_8O_4 = C_{13}H_{12}O_2N_4,$$

the sugar-like portion I in the above formula is split from the coloured structure II+III, which then represents the photo-flavin molecule. About the portion II little is known except that it is relatively stable and contains two weakly basic N-atoms which are probably of tertiary nature and share the chromophoric double bonds.

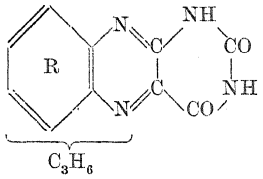
It may be tempting to associate the presence of the alloxan ring (III) with the function of the flavin molecule as a reversible redox system. This idea must however be abandoned. The alloxan-dialuric acid system as studied by Richardson and Cannan [1929] presents no analogies with the flavin system. Apart from the widely different potential values, the alloxan-dialuric acid system becomes unstable at $p_H > 5$, probably because of the isomerisation of alloxan into alloxanic acid. Furthermore the quinhydrone-like compound alloxantin is completely dissociated into alloxan + dialuric acid in the very range where semiquinone formation takes place in the flavin system. Whereas stabilisation of the alloxantin potentials in dilute solution requires the addition of iron salts, which leads to the formation of an iron complex acting as an electro-active intermediate between the components of the main system and the electrode [Hill and Michaelis, 1933], in the case of photo-flavin complex formation with an iron compound has been shown above to set up an irreversible system.

On alkaline hydrolysis the photo-flavin molecule is split into urea and an unknown acid: $C_{13}H_{12}O_2N_4 + 2H_2O \rightarrow CO(NH_2)_2 + C_{12}H_{12}O_3N_2$ [Warburg and Christian, 1933; Kuhn, Rudy and Wagner-Jauregg, 1933]. This acid yields on thermal decomposition $CO_2 + C_{11}H_{12}ON_2$. Kuhn and co-workers compare the formation of the acid $C_{12}H_{12}O_3N_2$ from the photo-product ("lumiflavin") with that of 2-hydroxyquinoxalin-3-carbonic acid from alloxazin:

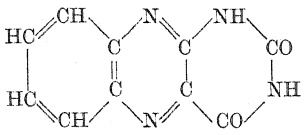
$$C_{10}H_6O_2N_4 + 2H_2O \rightarrow CO(NH_2)_2 + CO_2 + C_8H_6ON_2 \text{ [Kühling, 1891].}$$

Though Kuhn, after preliminary experiments, rejected the idea that the flavins may be constructed similarly to the alloxazin model [Kuhn and Wagner-Jauregg, 1933] he states in a later paper that on reduction in acid solution alloxazin yields a red intermediate which is similar to the flavin intermediate. It is known that even the simple quinoxalin shows the formation of a brightly coloured intermediate which is blue in this case, on reduction in acid solution [Hinsberg, 1884]. That the alloxan ring of the flavin should be contained in a purine skeleton is unlikely in view of the fact that the extinction coefficient of the short ultra-violet absorption band of the flavin is of a higher order of magnitude than that of any purine compound so far known (private communi-

cation from Dr E. R. Holiday). There seems therefore to exist some presumptive evidence in favour of a formulation of photo-flavin as follows:



This would place the flavins in the alloxazin series[1]. We find that the simplest representative of this class, namely, alloxazin itself,



exhibits even more similarities with photo-flavin than were mentioned by Kuhn. Its solution in dilute sodium carbonate is green-yellow and shows a green fluorescence. Addition of sodium hydrosulphite or titanous chloride causes slow decoloration. The red intermediate in mineral acid solution is formed not only by reduction with zinc but also by any strong reductant, *e.g.* chromous chloride. The red intermediate is obtained also on re-oxidation of the leuco-compound with air and shows a remarkable tendency to persist under aerobic conditions. A comparative study of the oxidation-reduction behaviour of alloxazin and photo-flavin and especially of the effect of substitution in the three ring systems on the potentials and on the spectrum is being undertaken in this laboratory.

The structural relationship of such a molecule as that suggested above to

Table IV.

Compound	Colour of Holoquinone	Colour of Semiquinone	E_0' at p_H 7
Hallachrome [Friedheim, 1933]	Red, $p_H < 8{\cdot}53$ Green $> 8{\cdot}53$	Brown (?)	+0·022 v.
α-Hydroxyphenazine [Michaelis *et al.* 1932]	Orange	Green	−0·07 v.*
Pyocyanine (α-hydroxy-*N*-methylphenazine) [Friedheim and Michaelis, 1931; Elema, 1931]	Red, $p_H < 4{\cdot}9$ Blue $> 4{\cdot}9$	Green	−0·034 v.
Chlororaphine (phenazine-α-carbonamide) [Elema, 1933]	Yellow	Green	−0·120 v.
Photo-flavin	Yellow	Red	−0·227 v.
Rosinduline 2 G [Michaelis, 1931, 3]	Red	Violet	−0·281 v.
Viologens (*NN'*-disubstituted γγ'-dipyridyliumchlorides) [Michaelis and Hill, 1933]	Colourless	Blue-violet	−0·4 v.

* From the diagrams reproduced by Michaelis *et al.* [1932, see also Michaelis 1933] it would appear that the E_0' of α-hydroxyphenazine at p_H 7·0 is −0·17 v. This value however refers to the calomel and not to the normal hydrogen electrode as stated in the text. [Private communication by Prof. L. Michaelis.]

[1] *Note added to proof.* This hypothesis has been rendered very probable by synthetic studies in the alloxazine series [Stern and Holliday (1934), *Ber. deutsch. chem. Ges.* **67**, 1104].

the phenazine pattern is quite obvious. Kuhn [1934] has already pointed out several analogies in their respective oxidation-reduction behaviours between the flavins and pyocyanine (α-hydroxy-*N*-methylphenazine). It occurs to the writer that an even more striking similarity exists between the flavins and rosinduline 2 G, especially in view of the fact that the normal potentials of this compound are close to those of photo-flavin (E_0' at p_H 7 −281 mv.) [Michaelis 1931, 3]. It should be mentioned however that the colour of the semiquinoid forms of simpler phenazine derivatives is green in contrast to the red semiquinone of photo-flavin. But the example of rosinduline where the semiquinone is violet demonstrates that the rule that the intermediates of phenazine compounds are green does not hold for the more complex derivatives.

This discussion may be concluded by a compilation of some properties of a few systems capable of semiquinone formation (Table IV).

Summary.

The behaviour of photo-hepatoflavin as an oxidation-reduction system has been studied by means of potentiometric analysis. Titrations were performed between p_H −0·2 and 12·4. Photo-hepatoflavin represents a perfectly reversible redox system, the normal potential E_0' of which is comparatively negative, namely −0·227 v. at p_H 7·0, referred to the normal hydrogen electrode.

In the neutral and alkaline ranges the titration curves are similar to those of a common organic redox indicator with an electron number of two. In the acid range the slopes of the curves become increasingly steeper with increase of $[H^+]$. At very low p_H values a break in the curves appears indicating that the reduction and oxidation are taking place here in two distinct steps each involving the exchange of one electron. The intermediate stage is characterised by a bright red colour (Kuhn). The observations point strongly to a semiquinone structure for this intermediate. A chart is given illustrating the relation between p_H and the normal potentials of the different forms (holoquinoid, semiquinoid, benzenoid form) of photo-flavin. All three forms show a fair degree of stability. The existence of appreciable amounts of the semiquinone is limited to the p_H range below 2.

The theory of Michaelis on two-step oxidation with intermediary semiquinone formation has been applied to the present system; and the distance of the normal potentials of the single steps from the mid-point found on titration, and also the effective formation constant of the semiquinone, have been calculated by means of Michaelis's equation.

Experiments carried out with the corresponding photo-product of the yeast-flavin (Warburg's yellow oxidation enzyme) yielded confirmatory evidence for the identity of the photo-flavins.

From this study several conclusions have been drawn concerning the structure of photo-flavin and especially as to the nature of the grouping of the molecule responsible for its properties as a reversible redox system. The inference is that the alloxan-dialuric acid equilibrium cannot correspond to the system set up by photo-flavin and its reductants, although the presence of an alloxan ring has been demonstrated in the molecule by Warburg and by Kuhn. A purine structure is likewise improbable. Presumptive evidence is brought forward for a structural relationship between photo-flavin and the alloxazin series as first discussed but later denied by Kuhn. This would explain certain common features of photo-flavin and phenazine derivatives, especially rosinduline, *e.g.* the negative range of potential and the phenomenon of semiquinone formation.

The author wishes to thank Prof. E. C. Dodds for his interest and encouragement. Furthermore he wishes to thank him and the Council of the Middlesex Hospital Medical School for the hospitality afforded. Sincere thanks are also due to Prof. L. Michaelis who was good enough to read the MS and to advance helpful criticisms.

REFERENCES.

Bierich and Lang (1934). *Z. physiol. Chem.* **223**, 180.
—— —— and Rosenbohm (1933). *Naturwiss.* **21**, 496.
Elema (1931). *Rec. Trav. Chim. Pays-Bas*, **50**, 807.
—— (1933). *Rec. Trav. Chim. Pays-Bas*, **52**, 569.
Ellinger and Koschara (1933). *Ber. deutsch. chem. Ges.* **66**, 1411.
Friedheim (1933). *Biochem. Z.* **259**, 257.
—— and Michaelis (1931). *J. Biol. Chem.* **91**, 355.
Greville and Maclagan (1931). *Trans. Faraday Soc.* **27**, 210.
Hill and Michaelis (1933). *Science*, **78**, 485.
Hinsberg (1884). *Ber. deutsch. chem. Ges.* **17**, 318.
Holiday (1930). *Biochem. J.* **24**, 619.
Kühling (1891). *Ber. deutsch. chem. Ges.* **24**, 2363.
Kuhn (1934). Lecture Heidelberg, 15th Jan.; rep. *Z. angew. Chem.* **47**, 105.
—— György and Wagner-Jauregg (1933). *Ber. deutsch. chem. Ges.* **66**, 576.
—— Rudy and Wagner-Jauregg (1933). *Ber. deutsch. chem. Ges.* **66**, 1950.
—— and Wagner-Jauregg (1933). *Ber. deutsch. chem. Ges.* **66**, 1577.
—— —— (1934). *Ber. deutsch. chem. Ges.* **67**, 361.
Michaelis (1931, 1). Handb. Pflanzenanalyse, **1**, 450.
—— (1931, 2). *J. Amer. Chem. Soc.* **53**, 2953.
—— (1931, 3). *J. Biol. Chem.* **91**, 369.
—— (1931, 4). *J. Biol. Chem.* **92**, 211.
—— (1932). *J. Biol. Chem.* **96**, 703.
—— (1933). Oxydations-Reduktions-Potentiale, 2nd ed. p. 107.
—— and Eagle (1930). *J. Biol. Chem.* **87**, 713.
—— and Hill (1933). *J. Gen. Physiol.* **16**, 859.
—— —— and Schubert (1932). *Biochem. Z.* **255**, 66.
Richardson and Cannan (1929). *Biochem. J.* **23**, 68.
Stern (1933). *Nature*, **132**, 784.
—— (1934, 1). *Nature*, **133**, 178.
—— (1934, 2). *Ber. deutsch. chem. Ges.* **67**, 654.
—— (1934, 3). *Tabulae Biologicae*, **4**, 1.
Warburg and Christian (1932). *Biochem. Z.* **254**, 438.
—— —— (1933). *Biochem. Z.* **266**, 377.
Weitz (1928). *Elektrochem.* **34**, 538.
Willstätter and Piccard (1908). *Ber. deutsch. chem. Ges.* **41**, 1458.

CXXXVI. STUDIES IN FAT METABOLISM IN THE FOWL.

I. THE COMPOSITION OF THE EGG FAT AND DEPOT FAT OF THE FOWL AS AFFECTED BY THE INGESTION OF LARGE AMOUNTS OF DIFFERENT FATS.

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The study of the formation and utilisation of fat in the fowl is of fundamental interest, since our knowledge of these processes in the bird is very meagre, investigations of fat metabolism having been so far carried out almost exclusively on mammals. With a view to eliciting information along these lines it was decided to investigate two problems which presented themselves as being of primary importance to the poultry industry. (1) The approximate composition of the mixed fatty acids of the normal egg and the extent to which this may be modified by the inclusion of fat in the ration. (2) The influence of ingested fat on the composition of the depot fat. The latter investigation is of a preliminary nature.

In the following paper the two problems outlined will be dealt with in separate sections.

SECTION I.

Effect of ingested fat on the composition of the egg fat.

The data available for the egg fat concern chiefly the neutral fats or the phosphatides and not the mixed fatty acids of the entire yolk. The ether extract of the fresh yolk is about 30–32 %, a figure that appears to remain relatively constant, but variable results have been obtained for the phosphatide content, *e.g.* Mangold [1931], 4 %; McCollum *et al.* [1912], 9·4 %; Manasse [1906], 9–10 %; Serono and Palozzi [1911], 11–12 %.

The discrepancy between these results is probably due to the fact that no satisfactory method for estimating the amount of phosphatide present has yet been evolved, but it is possible also that the phosphatide content may be dependent on certain variable factors.

Many workers have investigated the composition of egg-lecithin with regard to its different components. References to this are given by MacLean [1918] and Needham [1931]. Cousin [1903], working chiefly on commercial samples of egg-lecithin, estimated the proportions of the different fatty acids present as follows: palmitic, 28·5 %; stearic, 14·2 %; oleic, 33 %; linoleic, 24 %. Levene and Rolf [1922] and Hatakeyama [1930] both recorded the presence of arachidonic

acid, and stated that linoleic acid was present only in small amount. The former found that, in contrast to liver-lecithin, egg-lecithin contained only a small proportion of highly unsaturated acids. Sueyoshi and Furukubo [1931], working with lecithin carefully purified from fat and kephalin, found that the saturated acids consisted mainly of *iso*palmitic acid, with only small amounts of palmitic and stearic acids. The proportions of the unsaturated acids present, calculated from their bromination products, were as follows: oleic, 73·2 %; clupanodonic, 5·1 %; linoleic, 2·1 %.

The results obtained for the composition of the neutral fat by the earlier workers [Liebermann, 1888; Kitt, 1897; Serono and Palozzi, 1911], present wide variations and are subject to criticism on account of the technique employed. The only detailed quantitative analysis of the egg fat available is that of Grossfeld [1933], who examined the mixed fatty acids obtained from the egg by the following method. Egg-yolks were treated with warm hydrochloric acid and extracted with light petroleum. The solution was freed from acid and water and the oil obtained by evaporation. It contained 5·1 % of unsaponifiable material and 90·7 % of mixed fatty acids, of the following composition: higher saturated fatty acids (permanganate method) 34·6 %; solid saturated fatty acids (lead salt-alcohol method) 32·4 %; stearic acid 2·2 %; palmitic acid 32·4 %; oleic acid 44·2 %; linoleic acid 18·0 %; linolenic acid (Kaufmann) 3·2 %. Butyric and *n*-octanoic acids were absent.

Various investigators have ascertained that the composition of the egg fat is affected by feeding. Henriques and Hansen [1903] found that on a carbohydrate diet the iodine value of the neutral fat averaged 78·8. With linseed feeding it rose to 97 and with hempseed to 119–123. Although the neutral fat was affected by the feeding, the fatty acids of the lecithins remained unchanged. This finding was not confirmed by McCollum *et al.* [1912] who found that the iodine value of the neutral fats varied from 52 on an approximately lipoid-free diet to 64 on an ordinary ration, while the iodine value of the lecithins on the former was 35, and on the latter 63. Similar results were obtained by Terroine and Belin [1927].

It thus appears that there is comparatively little reliable information available as to the composition of the egg fat, and though it has been ascertained that it may be affected by the nutrition of the fowl, yet we have no knowledge with regard to the changes which take place in the proportions of the component fatty acids.

Experimental.

In the experiments to be described, Light Sussex hens were kept under ordinary commercial conditions with access to free range. The control ration, which was the ordinary stock ration used on the farm, consisted of bran, maize, sharps and Sussex ground oats, supplemented by 7 % extracted soya bean meal, 7 % fish meal, and 3 % mineral mixture. The experimental groups (with one exception) received the control ration with the addition of high percentages of fats of varying composition.

The effect of ingested fat was studied (*a*) on successive eggs of individual hens, alteration in iodine value being taken as indication of a change in composition, and (*b*) on composite samples of eggs taken from groups of birds receiving different types of fat in the ration.

Under (*a*) the effects of a relatively saturated fat, *viz.* mutton fat (incorporated in the control ration at a 28 % level) and a highly unsaturated oil, *viz.* hemp oil (fed in the form of hempseed alone) were investigated. The birds were trap-

nested, and the individual eggs collected. Each yolk was separated from the white as completely as possible and gradually diluted with distilled water, 10 ml. of alcohol and 5 ml. of 40 % KOH were added and the whole was made up to 200 ml. Aliquot portions of this were saponified with a further addition of alcoholic potash for 1½ hours in an evacuated pressure-flask on a steam-bath. The saponified solution was then treated according to the method described by Mottram [1910] for the estimation of the fatty acids of liver tissue. The mixed fatty acids were dried to constant weight in an oven through which a current of CO_2 was passing, and the iodine values were determined by Wijs's method. The changes occurring in the egg fat as shown by the iodine values are shown graphically in Fig. 1.

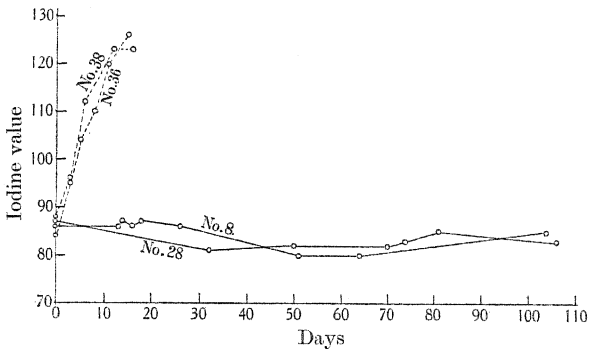

Fig. 1. Effect of ingestion of hemp oil and mutton fat on the mixed fatty acids of egg-yolk.
– – – – Hemp oil (I.V. = 164); ——— Mutton fat (I.V. = 45).

In section (*b*) the experimental rations were as follows:

(1) Fish meal-free mash
(2) Control mash } Normal rations.
(3) ,, +28 % palm kernel oil.
(4) ,, +28 % palm oil.
(5) ,, +28 % mutton fat.
(6) ,, +28 % linseed oil.
(7) ,, +28 % hemp oil.
(8) Hempseed alone.

The fish meal-free ration (which contained degermed maize, bran, whole yeast, rice, alfalfa and mineral mixture, with 7 % extracted soya bean meal) was fed in addition to the control mash, since it was thought that the fish meal contained in the latter might possibly influence to some extent the composition of the mixed fatty acids of the egg. The ether extract of the former was only 2·3 % as compared with 3·8 % in the control ration, as the constituents were selected for their low fat content. Analyses of the normal rations and of hempseed are given in Table I.

Table I. *Average composition of rations.*

	Moisture %	Ether extract %	Protein %	Carbohydrate %	Fibre %	Ash %
Control	12·5	3·8	14·1	57·9	6·6	5·1
Fish meal-free	10·9	2·3	13·7	67·8	1·3	4·0
Hempseed	8·5	29·5	24·3	15·0	17·9	6·7

Rations 3–8 represent wide differences in the composition of the ingested oils, those in groups 3–5 having a solid acid content ranging from 80 % in the palm kernel oil to 46 % in the palm oil, with unsaturated acids consisting mainly of oleic acid, while in 6, 7 and 8 the percentage of solid acids varies from 6 to 9 %, and the unsaturated acids contain a large proportion of linoleic and a considerable amount of linolenic acid. Analyses of the oils are given in Table II.

Table II.

Composition of oils.

		Hemp oil	Linseed oil	Palm oil	Mutton fat
Oil	I.V.	163·7	180·6	53·1	44·5
	Sap. no.	190·4	191·7	196·0	196·3
	Mol. wt.	294·7	292·6	286·2	285·8
	CNS value	93·1	112·8	—	—
Mixed acids	I.V.	172·9	191·54	55·0	46·1
	Mol. wt.	283·0	282·2	273·8	277·0
	% solid acids	6·1	9·2	46·3	51·0
Liquid acids	I.V.	183·0	206·5	103·8	89·2
	Mol. wt.	280·3	282·0	283·4	276·0
	CNS value	104·7	131·4	—	—
	Percentage composition of mixed fatty acids.				
	Solid acids	6·1	9·2	46·3	51·0
	Oleic acid	11·9	11·41	46·4	46·8
	Linoleic acid	67·2	38·98	7·3	2·2
	Linolenic acid	14·8	40·41	—	—

Palm kernel oil was not examined, but according to Collin and Hilditch [1928] its component fatty acids are: myristic, lauric and lower saturated acids, 70·7 %; palmitic, 8·8 %; stearic, 1·3 %; oleic, 18 %; with only a trace of linoleic acid.

Samples consisting of 10–12 eggs were collected from the different groups after at least 6–8 weeks from the commencement of feeding the experimental rations. The yolks were separated from the whites and ground up with anhydrous sodium sulphate. This material was then extracted in a Bolton and Revis extractor for prolonged periods with anhydrous ether, the solvent being frequently changed, until on evaporation no residue remained. The egg oil thus obtained, which was viscid and orange-coloured, was dried *in vacuo* at 100° and when not analysed immediately was put into small ground stoppered bottles and stored in the dark at −20°.

No attempt was made to separate the neutral fats from the phosphatides, owing to the great difficulty of obtaining either of them in a pure state without admixture with the other. Since the oil obtained by the method just described yielded mixed acids the iodine value of which was only slightly lower than that of the mixed acids obtained by the Mottram saponification method, it was considered that all except very small amounts of the total fatty acids derived from both the neutral fats and the phosphatides had been extracted, and it is probable therefore that the analyses given are representative of the total fatty acids of the egg-yolk. This view is supported by the fact that our results for normal eggs agree well with those of Grossfeld [1933], who, by his method of heating the yolk with hydrochloric acid, undoubtedly obtained the phosphatide-fatty acids as well as the fatty acids of the neutral fat in his mixed acids.

The oil obtained by the extraction method was saponified, the unsaponifiable matter removed, the mixed fatty acids precipitated with hydrochloric acid, taken up in light petroleum and dried *in vacuo* at 100°.

Determinations were made of the iodine value and molecular weight of the mixed acids. The solid and liquid acids were separated by crystallisation of the lead salts from 95 % alcohol [Twitchell, 1921], using the procedure of Hilditch and Priestman [1931]. The iodine value of the solid acids was estimated and the percentage of solid acids corrected for any oleic acid present.

The solid acids always contained traces of a brownish non-fatty material, which did not dissolve during the second precipitation of the lead salts. The molecular weights therefore are probably slightly too high.

The alcoholic solution of the liquid acids was diluted to twice its volume, the liquid acids separated with dilute acetic acid and taken up in light petroleum. The ethereal solution was repeatedly washed till free from acid, the ether evaporated off and the acids dried *in vacuo* at 100°.

The iodine value, molecular weight and thiocyanogen value (CNS value) of the liquid acids were determined. The percentage of hexabromides was estimated in a few cases, but did not prove satisfactory, and it was decided to substitute the thiocyanogen value as a means of estimating the linolenic acid, information having been obtained that this method gave higher and more accurate results [Griffiths and Hilditch, 1934]. Determinations were carried out, with careful attention to the absolute dryness of all reagents and apparatus employed, as recommended by Kaufmann and Keller [1929].

Oleic and linoleic acids were calculated from the iodine value of the mixed acids after allowance had been made for the solid acids and linolenic acid present. It was assumed that acids more unsaturated than linolenic acid were absent, though this was probably not strictly true.

Results of the analyses of the mixed fatty acids of the egg fat are given in Table III.

Table III.

Composition of mixed fatty acids of egg-yolk.

		Control mash	Fish meal-free mash	Mash + palm kernel oil	Mash + palm oil	Mash + mutton fat	Mash + linseed oil	Mash + hemp oil	Hempseed alone
Mixed acids	I.V.	84·4	80·0	80·5	85·9	84·0	123·1	115·7	127·2
	Mol. wt.	280·5	283·0	280·3	281·4	284·0	281·8	282·1	280·6
Solid acids	%	31·4	31·2	30·3	27·8	29·5	23·9	24·3	21·4
	Mol. wt.	263·5	264·0	266·4	265·5	268·0	265·7	267·4	266·0
Liquid acids	I.V.	121·2	115·9	115·0	120·0	117·7	161·3	156·3	162·8
	Mol. wt.	278·2	284·0	278·8	280·0	283·3	283·4	281·1	278·9
	CNS value	94·0	93·3	92·3	90·8	93·7	111·2	96·4	101·8

Percentage composition of mixed fatty acids.

Solid acids	31·4	31·2	30·3	27·8	29·5	23·9	24·3	21·4
Oleic acid	46·7	51·4	51·9	49·8	50·8	33·8	28·8	26·7
Linoleic acid	19·0	15·0	16·1	21·7	16·9	24·9	41·7	41·9
Linolenic acid	2·9	2·4	1·7	0·7	2·8	17·4	5·2	10·0

Discussion.

No effect on the flavour of the egg was observed during the feeding of the experimental rations, except in the case of linseed oil, which produced an unpleasant taste in the soft-boiled egg.

The results obtained on successive eggs of individual hens show that on the hempseed ration the mixed fatty acids became steadily more unsaturated, the normal iodine value of 84–88 being increased to 123–126 at the end of 16 days, and there remaining constant, showing that by this time all the layers of yolk

(which are laid down concentrically) had been deposited under the influence of the hempseed diet. The period required for reaching the maximum iodine value is a measure of the period required for the growth of the ovum. This is not necessarily a constant, but will differ in different individuals, or even in the same individual at different seasons, according to the rate of production.

Compared with hempseed, mutton fat had little effect on the iodine value of the mixed acids. A slightly lower value was obtained from the 7th to the 10th week after the commencement of the period, but towards the end it returned to the normal.

It appears therefore that while unsaturated oils given in the form of a diet such as hempseed readily affect the composition of the yolk fat, as indicated by the change in iodine value, the ingestion of a hard fat, *viz.* mutton fat, in conjunction with a cereal ration, does not significantly affect it, even after prolonged feeding.

Reference to Table III shows that on a cereal diet with a protein supplement, the degree of saturation of the mixed acids of the egg is similar to that of the mixed acids of the body fat on a normal ration. Solid acids constitute about 31 %, and according to the molecular weight (263·7) appear to consist mainly of palmitic acid (molecular weight 256).

Oleic acid is the main constituent of the liquid fraction, with smaller amounts of linoleic and 2–3 % of linolenic acid, as determined by the thiocyanogen value. The percentages of linoleic (15–19 %) and of oleic acid (47–51 %) are of the same order as those found by Grossfeld [1931] in hen depot fat. The composition of the mixed fatty acids from the control and fish meal-free eggs is very similar to that recorded by Grossfeld [1933] for commercial eggs of Danish origin.

Since the fish meal-free ration contained only 2–3 % of ether extract, it is evident that a considerable proportion of the normal egg fat must be derived by synthesis from the non-fatty constituents of the food rather than by direct assimilation of fat. It cannot be definitely stated whether synthesis of linolenic acid from carbohydrate takes place or not, since both the control and fish meal-free rations contained soya bean meal, which, even when solvent-extracted, may contain traces of this acid.

The addition of palm kernel oil to the ration produced a fatty acid mixture of the same composition as that of the fish meal-free eggs. No lowering of the molecular weight of the mixed acids or of the iodine value of the liquid acids was observed, as would have been the case had appreciable amounts of the lower saturated acids been deposited.

The ingestion of palm oil and mutton fat did not affect the composition to any significant extent, though in the former case the percentage of linolenic and of solid acids was slightly reduced, and that of the linoleic acid slightly increased.

With the feeding of unsaturated oils a marked increase in the degree of unsaturation took place, accompanied by a decrease in the percentage of solid acids, a marked decrease in oleic acid and an increase in both linoleic and linolenic acids.

The iodine value of the mixed acids was highest, and the percentage of solid acids lowest, on the hempseed diet, *i.e.* where the ration fed, though rich in unsaturated fatty acids and protein, was relatively deficient in carbohydrate. When the hemp oil was fed in conjunction with a carbohydrate-rich ration, the degree of unsaturation was reduced and the percentage of solid acids slightly raised, as compared with the hempseed diet. The mash *plus* linseed oil produced a greater degree of unsaturation in the mixed acids than the mash *plus* hemp oil,

in accordance with the iodine values of the respective oils. The percentage of solid acids was the same in both cases.

The percentage of linoleic acid appears to be related to some extent to that of the ingested oils. The linoleic acid content of the mixed acids of the oils, and of the mixed acids of the eggs produced on the rations containing them, are as follows: hemp oil, 67 %; hempseed and hemp oil eggs, 42 %; linseed oil, 39 %; linseed eggs, 25 %; mutton fat and palm oil, 2–7 %; mutton fat and palm oil eggs, 17–22 %. Even when no added linoleic acid is present in the diet the linoleic content of the egg fatty acids will be approximately 15–19 %.

The linolenic acid content also tends to run parallel with that of the ingested oils, the largest amount being found when linseed oil is fed, the percentage here being over five times that found in the normal egg. Hempseed fed alone raised the percentage of linolenic acid in the mixed acids to three times that of the normal egg. In this case the linolenic acid content of the liquid acids, *viz.* 12·7 %, approximates to that present in the oil itself.

When the diet contained ample carbohydrate in addition to the hemp oil, however, the linolenic acid was reduced by 50 %. To what extent the linolenic acid content might have been raised on linseed alone could not be determined, as, owing to its unpalatability, fowls can rarely be induced to eat more than small amounts of the seed.

From these results it appears that the hen, even when abundant carbohydrate is present for the synthesis of normal fat, will utilise ingested fatty acids for direct deposition in the egg fat. When the ration containing the unsaturated fatty acids is deficient in carbohydrate, a greater proportion of the acids will be deposited. On the other hand, in spite of the ingestion of large amounts of saturated acids in the presence of ample carbohydrate, the composition of the mixed fatty acids of the egg tends to remain similar to that produced on a cereal *plus* protein ration containing 2–4 % of ether extract.

It seems possible that in the synthesis of egg fat, the hen exercises a selective influence, and there may be a degree of saturation of the fat which cannot be increased by the ingestion of saturated fatty acids, but which can be reduced to a marked extent by the assimilation of unsaturated acids.

Since the fat of the egg-yolk serves as a source of energy for the developing embryo, the fact that its composition can be materially influenced by certain nutritional factors may be of considerable significance in connection with embryonic nutrition, hatchability, *etc.*

SECTION II.

Effect of ingested fat on the composition of the depot fat.

The dependence of the constitution of the fat reserves on the composition of the diet has been clearly established in the case of the pig, notably by Ellis and co-workers [1926; 1930; 1931] in their extensive studies on soft pork, and by Bhattacharya and Hilditch [1931], and in the case of the rat by Anderson and Mendel [1928], Eckstein [1929], Reed *et al.* [1930], Banks *et al.* [1933] and others. All these investigators have found that dietary fat may exert a marked effect on the composition of the depot fat.

Little attention however has been directed towards the study of fat formation in birds. Quantitative data exist for the composition of goose fat [Bömer and

Merten, 1922], and for the fat of the hen [Grossfeld, 1931], but the effect of diet on such composition has received little consideration.

Henriques and Hansen [1903] found that the iodine values of the fat of three hens fed on a diet of hempseed were 103, 120 and 135 respectively. Schirmer [1921] records an iodine value of 74·8 for the fat of geese on a diet of maize and olive oil, and 63·2 on maize and mutton fat. In spite of the statement in a report from the Kansas Agricultural Experiment Station [1922] that "none of the short chain fatty acids of the food are deposited in the body fat of the hen, while the unsaturated fatty acids are so deposited" the actual tabulated data show clearly that either saturated or unsaturated fats can be deposited in the fat depots of the hen.

Experimental.

The birds in this section of the experiment were kept under the conditions of management already described. The same control ration was employed.

The fats investigated, *viz.* palm kernel oil, mutton fat and hemp oil, provided examples of fats differing considerably in composition from that of the normal body fat. Since the inclusion of small amounts of fat in the ration only slightly affected the normal composition of the body fat in the pig, it was decided to incorporate the palm kernel oil and mutton fat in the control ration at a high level, *viz.* 28 %. In investigating the effect of hemp oil, hempseed was fed as the sole diet, owing to the difficulty of obtaining sufficient quantities of the oil

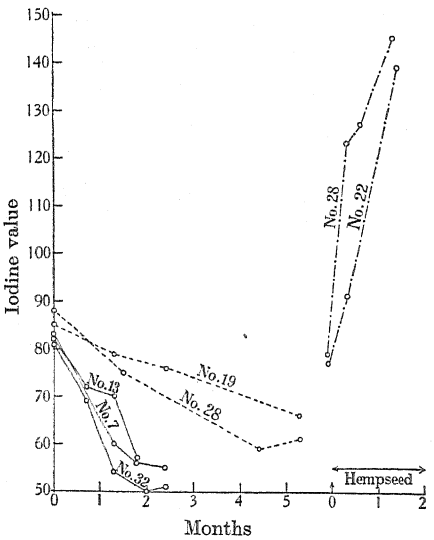

Fig. 2. Effect of ingestion of palm kernel oil, mutton fat and hemp oil on the mixed fatty acids of abdominal fat.

——— Palm kernel oil (I.V. = 15); – – – – Mutton fat (I.V. = 45);
•—•—• Hemp oil (I.V. = 164).

itself. The percentages of fat in these three diets were very similar, since the hempseed contained 28–29 % of ether extract. It was however richer in protein and considerably poorer in carbohydrate than the fat *plus* mash rations.

The work of Scott [1920] having shown that considerable individual variation in the composition of the body fat occurs in pigs even under the same

conditions of management and dietary régime, it was decided to study progressive samples of body fat obtained from the same individual during the experimental period.

Samples of fat (0·5–0·8 g.) could be readily removed without injury to the bird from the abdominal region, where there is a comparatively large superficial deposit in a well-nourished hen. These were taken while the birds were still on the control ration, and again at intervals during the experimental period. The fat was saponified in evacuated pressure-flasks and the iodine value determined on the mixed fatty acids. The results are shown graphically in Fig. 2.

In pigs and cattle it has also been found that the composition of the fat varies significantly in different areas, therefore samples from the various fat depots were examined to ascertain whether such variation in composition occurs in the fowl. The results are shown in Tables IV and V. As the iodine values of the different areas were very similar, it was assumed that the abdominal layer could safely be taken as representative of the body fat as a whole.

Table IV. *Iodine values of mixed fatty acids of fat from different depots.*

No. of hen	Gizzard	Neck	Leg muscle	Abdominal
3	93	92	93	90
15	79	79	—	78
17	64	67	65	65
25	90	90	90	88

Table V. *Iodine values of mixed fatty acids of fat from different depots.*

No. of hen	Skin	Neck	Leg muscle	Abdominal	Gizzard	Kidney	Mesenteric	Epicardial	Av. I.V.	Max. diff. between depots
5	87·3	88·1	86·2	87·1	84·5	84·4	84·0	85·6	85·9	4·1
8	81·4	78·0	—	—	75·6	77·7	—	—	78·2	5·8
29	92·0	89·98	88·4	88·1	89·0	87·8	88·0	90·2	89·2	4·2
32	85·9	81·7	81·2	80·8	79·7	81·6	80·7	85·7	82·2	6·2

Discussion.

Composition of different fat depots.

In the hen fat is deposited as a thick abdominal layer and as a layer surrounding the gizzard. Smaller deposits are found in the neck region, chiefly between the clavicles, and also along the ventral muscles of the femur. The last-mentioned two deposits are relatively superficial.

Table IV shows that the iodine values of the fatty acids of the main fat depots are very similar. Since this is in contrast to the findings in pigs and cattle, examination of the smaller depots was made, with the results shown in Table V. The skin fat was obtained by extraction of the skin itself, which contains a certain amount of fat along the tracts of the feather follicles. The gizzard fat was obtained from the dorsal side of the gizzard only, as the fat on the ventral side is more or less continuous with the abdominal layer. The differences in iodine value are slightly greater than in Table IV but are nevertheless much smaller than those recorded for pigs and cattle. In two cases the skin is the most unsaturated layer, in one the epicardial fat is equally high, in the fourth the neck fat is one unit higher. It is evident that the most deep-seated layers, *viz.* kidney, mesenteric and epicardial fat, do not show a decidedly lower iodine value than the subcutaneous fat, as recorded by Henriques and

Hansen [1901] in cattle, where the iodine value of the skin fat was 52, and that of the mesenteric fat 40. Even greater differences were found by Bhattacharya and Hilditch [1931] in pigs on a normal ration, where the iodine value ranged from 63 in the outer back fat to 46 in the perinephric fat. The various fat depots of the hen therefore, judged by the iodine values, appear to be of more uniform composition than is the case in certain mammals. That the uniformity extends also to the component fatty acids has been shown by the detailed analyses of Hilditch *et al.* [1934]. This fact lends support to the suggestion that the temperature of the different areas is not the sole factor in determining the composition of the fat reserves [Dean and Hilditch, 1933].

The variation between individuals in Table IV is due to the fact that the birds were taken from the fat-fed groups at various times. No. 8 (Table V) had also received a ration containing mutton fat for 6 weeks. Nos. 5, 29 and 32, however, which were all on the control ration, show that some individual variation in composition exists, even under identical nutrition and management, as is the case also in pigs [Scott, 1920].

Effect of ingested fat on the composition of the body fat.

In Fig. 2 individual variation is again evident in the five hens examined at the commencement of the experimental period, while they were still on the control ration, the iodine values ranging from 81 to 88. A steady change towards a harder fat occurred when rations containing 28 % of mutton fat and of palm kernel oil were fed. Individuals however varied slightly in rate and degree of response to the diets. For example, in the case of No. 19 the mutton fat ration lowered the iodine value from its original level (85) to 66 at the end of about 5 months, while in No. 28 the iodine value fell from 88 to 59 in 4½ months. After this the iodine value of No. 28 appeared to continue at practically the same level. Three other hens (not shown in the figure) on the mutton fat mash were operated on, and it was evident from observation of the birds that the individual response to the feeding depended in a large measure on the physiological condition of the bird (whether laying, non-laying or broody) and the resulting capacity for food consumption. The iodine value in No. 28, whose food intake was very high, fell lower than in any of the other birds examined.

The birds on the palm kernel oil mash showed a marked response to the ingestion of this oil, the iodine value falling from the original level (81–83) to 51–55 in a little over 2 months. The more rapid change in this case was probably due to two causes. (1) The iodine value of the palm kernel oil, *viz.* 15, is much farther removed from that of the body fat than is that of mutton fat, *viz.* 45, hence the ingestion of equal amounts of the two fats would result in a greater decrease in iodine value in the case of the palm kernel oil feeding. (2) The palm kernel oil mash was obviously much more palatable to the birds than the mutton fat mash, and food consumption was therefore greater.

Four birds in the hempseed group were operated on. In each case a rapid and pronounced change in the direction of increased unsaturation took place. The results for two of the birds are shown in Fig. 2. No. 28 showed the most rapid and most marked response, the iodine value of 79 at the commencement of the period being raised to 145 within 6 weeks. In No. 22 the iodine value rose to 139, and in the other two birds to 124 and 130 respectively within the same period.

With regard to these data it should be noted that though the fat content of the diet was approximately the same in the three groups, the birds on hemp-

seed were receiving a ration which was considerably richer in protein but contained only a third of the carbohydrate content of the fat *plus* mash rations. In consequence, they probably tended to lay down the ingested fat more rapidly than if supplied with a ration containing an adequate amount of carbohydrate from which the normal body fat could be synthesised. The results show clearly, as did the tabulated data of the Kansas Agricultural Experiment Station Report [1922] that the degree of saturation of the mixed fatty acids of the body fat can be either increased or decreased by the ingestion of appropriate fats in the ration.

The record of the variations in the iodine value of the mixed fatty acids of the abdominal fat of No. 28 over the 12-month period during which she was under experiment seems worthy of note (see Table VI).

Table VI. *Changes in iodine value of mixed fatty acids of abdominal fat of No. 28.*

Date	I.V.		Date	I.V.	
—	88	Control ration	10. xii. 31	123	Hempseed ration
1. vi. 31	—	Mutton fat ration	17. xii. 31	127	,,
14. vii. 31	75	,,	9. i. 32	145	,,
8. x. 31	59	,,	16. i. 32	—	Control ration
10. xi. 31	61	,,	28. i. 32	139	,,
—	—	Control ration	10. ii. 32	137	,,
30. xi. 31	79	—	3. iii. 32	115	,,
1. xii. 31	—	Hempseed ration	2. vi. 32	90	,,

It will be seen that saturation has taken place more slowly than unsaturation, both during the production of a more saturated fat than the normal, and in the production of a normal fat from an abnormally unsaturated one. This does not necessarily mean that the hen tends to select the unsaturated acids more readily than the saturated acids, since various factors may have combined to produce this effect. The palatability of the respective rations, the ratio of fat to carbohydrate present, the degree of divergence of the iodine value of the ingested fat from that of the body fat, the varying appetite and condition of the bird, must all be taken into account.

The rapidity with which the experimental rations have influenced the composition of the body fat is not very surprising in view of the hen's intense physiological activity. In the fowl, the weight of thyroid per kg. of body weight, and also the percentage of iodine in the thyroid are greater than in mammals [Cruickshank, 1929]. A higher metabolic rate therefore results, as shown by the body temperature (104–108° F. as compared with 101° F. in the cow, or 103° F. in the pig). Respiration and heart beat, digestion and absorption are more rapid, while food consumption per unit of body weight is considerably greater. The high metabolic rate is essential in a system of reproduction entailing a rapid transformation of raw materials into food constituents for embryonic use. In a hen laying 250 eggs *per annum*, this involves an output of 31 lbs. of eggs, of which 4·1 lbs. are protein, 3·3 lbs. are fat and 3·1 lbs. are $CaCO_3$ in the form of shell.

From our results it appears that the selection which the hen exercises with regard to the deposition of ingested fat in the egg does not hold in the case of the body fat, which, so far as can be ascertained by the iodine value, may be markedly influenced by the fatty acids ingested, irrespective of their degree of saturation. It seems possible also that changes in the composition of the fat can be more rapidly induced in the bird than in the mammal, owing to the higher rate of metabolism of the former and the resulting rapid utilisation of food.

SUMMARY.

Effect of ingested fat on the composition of the egg fat. On normal cereal rations containing protein supplements, the mixed fatty acids of the egg fat contained about 31 % solid acids, 47–51 % oleic acid, 15–19 % linoleic acid and 2–3 % linolenic acid.

While the degree of saturation and the proportion of the component fatty acids could be considerably modified by the ingestion of unsaturated fatty acids, the ingestion of saturated acids had relatively little effect in altering the normal composition of the mixed fatty acids of the egg fat.

Effect of ingested fat on the composition of the depot fat. The iodine values of the mixed fatty acids of the different fat depots in the hen were determined. These indicated that the superficial and internal fat reserves are more uniform in composition than in the case of pigs and cattle.

Observations on individual birds showed that the ingestion of high percentages of saturated fatty acids in the form of palm kernel oil and mutton fat definitely decreased the degree of saturation of the mixed fatty acids of the depot fat, while the ingestion of unsaturated acids in the form of hempseed resulted in a marked and rapid increase in unsaturation.

I am greatly indebted to Mr E. T. Halnan for his interest and encouragement throughout the work. My best thanks are also due to Prof. T. P. Hilditch, whose advice and helpful criticism have been invaluable. The expenses of the investigation have been defrayed by a grant from the Empire Marketing Board.

REFERENCES.

Anderson and Mendel (1928). *J. Biol. Chem.* **76**, 729.
Banks, Hilditch and Jones (1933). *Biochem. J.* **27**, 1375.
Bhattacharya and Hilditch (1931). *Biochem. J.* **25**, 1954.
Bömer and Merten (1922). *Z. Unters. Nahr. Genussm.* **43**, 101.
Collin and Hilditch (1928). *J. Soc. Chem. Ind.* **47**, 261.
Cousin (1903). *Compt. Rend. Soc. Biol.* **55**, 913.
Cruickshank (1929). *Biochem. J.* **23**, 1044.
Dean and Hilditch (1933). *Biochem. J.* **27**, 1950.
Eckstein (1929). *J. Biol. Chem.* **81**, 613.
Ellis and Isbell (1926). *J. Biol. Chem.* **69**, 239.
—— and Zeller (1930). *J. Biol. Chem.* **89**, 185.
—— Rothwell and Pool (1931). *J. Biol. Chem.* **92**, 385.
Griffiths and Hilditch (1934). *J. Soc. Chem. Ind.* **53**, 75 T.
Grossfeld (1931). *Z. Unters. Lebensm.* **62**, 553.
—— (1933). *Z. Unters. Lebensm.* **65**, 311.
Hatakeyama (1930). *Z. physiol. Chem.* **187**, 120.
Henriques and Hansen (1901). *Skand. Arch. Physiol.* **11**, 151.
—— —— (1903). *Skand. Arch. Physiol.* **14**, 390.
Hilditch, Jones and Rhead (1934). *Biochem. J.* **28**, 768.
—— and Priestman (1931). *Analyst*, **56**, 354.
Kansas Agric. Exp. Sta. Biennial Rep. (1922), 17.
Kaufmann and Keller (1929). *Z. angew. Chem.* **42**, 20, 73.
Kitt (1897). *Chem. Zentr.* **68**, 1131.
Levene and Rolf (1922). *J. Biol. Chem.* **51**, 507.

Liebermann (1888). *Pflüger's Arch.* **43**, 71.
MacLean (1918). Lecithin and allied substances. (Longmans, Green and Co., London.)
McCollum, Halpin and Drescher (1912). *J. Biol. Chem.* **13**, 219.
Manasse (1906). *Biochem. Z.* **1**, 246.
Mangold (1931). *Arch. Geflügelk.* **5**, 286.
Mottram (1910). *J. Physiol.* **40**, 122.
Needham (1931). Chemical embryology. (Cambridge Univ. Press.)
Reed, Yamaguchi, Anderson and Mendel (1930). *J. Biol. Chem.* **87**, 147.
Scott (1920). *Florida Agric. Exp. Sta. Bull.* No. 157.
Schirmer (1921). *Arch. exp. Path. Pharm.* **89**, 263.
Serono and Palozzi (1911). *Chem. Zentr.* **82**, 772.
Sueyoshi and Furukubo (1931). *J. Biochem.* **13**, 155, 177.
Terroine and Belin (1927). *Bull. Soc. Chim. Biol.* **9**, 12.
Twitchell (1921). *J. Ind. Eng. Chem.* **13**, 806.

CXXXVII. BILE PIGMENTS. VI. BILIVERDIN, UTEROVERDIN AND OOCYAN.

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(*Received April 30th, 1934.*)

By dehydrogenation of bilirubin with ferric chloride a green pigment, dehydrobilirubin, has been prepared. The same pigment is found in nature as oocyan in the egg-shells of gulls and other birds [Lemberg, 1931] and as uteroverdin in the dog's placenta [Lemberg and Barcroft, 1932]. Dehydromesobilirubin, which differs from dehydrobilirubin merely in having saturated side-chains, was prepared by Fischer *et al.* [1932] and called glaucobilin.

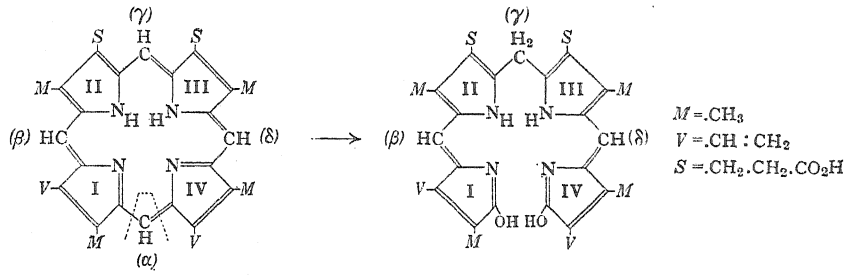
Analysis of the dimethyl esters of these pigments showed that these compounds possess two hydrogen atoms less than bilirubin and mesobilirubin, but the same number of oxygen atoms, *i.e.* six. The first analytical values of the oocyan ester showed some divergence, but later analyses [Lemberg, 1932] gave the same results for oocyan and dehydrobilirubin esters. The constitution of dehydrobilirubin is established in the present paper by analyses of dehydrobilirubin (free acid). The first analyses of this substance favoured a formula with eight oxygen atoms, while the results of Fischer on glaucobilin (free acid) corresponded to a formula with seven oxygen atoms. Analyses of carefully purified dehydrobilirubin establish the formula $C_{33}H_{34}O_6N_4$. The analyses of the free acid and the esters show furthermore that only two hydrogen atoms have been removed by the dehydrogenation of bilirubin.

A second point which required further investigation was the relation of dehydrobilirubin and dehydromesobilirubin to biliverdin and mesobiliverdin, pigments showing similar colours and absorption spectra, which are obtained by autoxidation of bilirubin and mesobilirubin in alkaline solution. It is now shown that "mesobiliverdin" is actually a mixture consisting largely of dehydromesobilirubin together with certain by-products which cannot be obtained in a pure state, but give the impression of being resinified substances. The yield of pure dehydrobilirubin from "biliverdin" is lower. This is to be expected as the unsaturated side-chains form a point of attack for alkali and oxygen. It seems preferable now to apply the convenient name "biliverdin" to pure crystalline dehydrobilirubin, instead of to a mixture of the latter with products of secondary alteration. The name mesobiliverdin rather than dehydromesobilirubin or glaucobilin will in future be used for the product with saturated side-chains.

A further simplification is rendered possible by the evidence now brought forward that the differences in optical properties between crystals of uteroverdin ester and oocyan ester, observed by Bernal in 1931 [Lemberg and Barcroft, 1932], are due merely to differences in growth and formation of the crystals, and probably do not indicate structural difference. Further investigation has confirmed the existence of two different crystal forms of biliverdin ester differing only with regard to pleochroism and extinction. One of these forms is obtained from biliverdin synthesised from pure bilirubin, the other (usually mixed with

the first) from biliverdin obtained from bilirubin mother-liquors and from the natural biliverdins, particularly uteroverdin.

This matter has been carefully investigated, as the existence of slight differences between isomeric bile pigments might have an important and interesting significance. According to recent investigations of Fischer and his collaborators bilirubin is represented by a formula in which four pyrrole nuclei are linked together by three carbon atoms to form an open chain. Bilirubin arises from protoporphyrin by oxidative opening of the porphyrin nucleus; one of the four CH-groups of the ring (α in the formula) is removed and replaced by two hydroxyl groups:



Fischer and Hess [1931] had assumed a symmetrical position of the methyl and vinyl groups in the rings I and IV of bilirubin (CH_3 next to OH in ring IV, instead of vinyl as in the formula above). This would have meant that the formation of bilirubin from protoporphyrin implies decomposition of the latter and resynthesis, as the position of the side-chains in ring IV could not be changed in any other way. Later, however, Siedel and Fischer [1933] proved that the side-chains of bilirubin are unsymmetrically arranged exactly like those in haemin and protoporphyrin. The ring is opened between the rings I and IV and the α-CH-group is removed.

Now three other bilirubins may arise from protoporphyrin if the ring could be opened at one of the other CH-groups (β, γ, δ) instead of at α. Thus isomeric bilirubins would originate differing from each other in the position of the side-chains attached to the rings. Such isomerides are often extremely similar and may even give no depression of the melting-point if mixed. Examples are found in the paper of Siedel and Fischer [1933] and previous ones of Fischer, *e.g.* the three mesobilirubins (IIIα, IXα, XIIIα) differing in the position of the side-chains attached to the rings I and IV. The difference of the two forms of biliverdin ester, if actually due to a structural difference, could only be caused by a difference in the position of the side-chains. The absence of a melting-point depression, the analytical data, the identity of the absorption curves and of other properties (such as basicity and solubility) leave no doubt that the nuclear system of the four pyrrole rings and the arrangement of the double bonds are the same in both forms.

The biliverdin ester of the form predominantly present in uteroverdin (which is different from the form obtained from pure bilirubin) can be obtained from mother-liquors of bilirubin and from gallstones. If thus structurally different types of biliverdin exist, then it is almost certain that there are two corresponding structurally different bilirubins. Uteroverdin, moreover, is formed in haemorrhages in placental tissue and is definitely of extrahepatic origin; thus the different biological origin is possibly connected with the chemical difference.

In the crystallographic investigations of the esters and their hydrochlorides I had the valuable help of Mr Rawlins of the Mineralogical Laboratory at Cambridge. These investigations show that the assumption of a structural difference between the two different crystalline forms is unnecessary, and that uteroverdin, oocyan and biliverdin may be considered identical. These investigations do not necessarily exclude the above explanation of the observed differences, but they give no evidence for it. Two different forms of protoporphyrin crystals have been observed by Richter [1930], one of which is caused by impurities according to Fischer *et al.* [1931] and Hamsik [1931][1].

The microanalytical part of the work has been done by Dr Roth, Heidelberg.

Experimental.

Mesobiliverdin by autoxidation of mesobilirubin.

The autoxidation of mesobilirubin to mesobiliverdin in alkaline solution was studied under different conditions and the product of the reaction was isolated. The effect of piperidine was tried, as this substance accelerates the autoxidation of dihydropolyenes [Kuhn and Drumm, 1932].

Five small crystallising dishes were filled as follows: I, 1·7 ml. 0·22 *N* NaOH. II, 3 ml. of the NaOH and 1 ml. water. III, IV and V like II but with the addition of 0·1 ml. *M*/10 ferrous sulphate solution to III, 0·01 ml. of the same solution to IV and one drop of piperidine to V. In each of them 0·1 g. of mesobilirubin was dissolved requiring theoretically 1·54 ml. of the NaOH solution (2 mol.). I contained 2·2, II–V 3·9 mols. of alkali. The dishes were placed in a water-filled desiccator and kept in it at 30°. After 12 hours only the solution in IV had turned green, the other ones were olive-brown. After 4 days' standing I and IV were green; II, III and V olive-green. Therefore the reaction proceeds best if a great excess of alkali is avoided. The reaction is accelerated by traces of iron, but not by piperidine.

The solutions I and IV were mixed and acidified with dilute acetic acid. The pigment (from 0·2 g. mesobilirubin) was extracted with ether, and after evaporation of the ether, the residue was extracted with methanol, which left some unchanged mesobilirubin undissolved. The mesobiliverdin was then esterified by passing in hydrogen chloride. After keeping overnight, esterification was completed by boiling the solution, diluted previously with half its volume of absolute methanol, for 15 minutes. It was then evaporated *in vacuo*. The residue was dissolved in a small amount of methanol, sodium acetate solution was added and the ester was extracted with ether. The extract was washed with 1 % sodium carbonate solution and water. The ether was evaporated and the product recrystallised from methanol. 31 mg. of pure mesobiliverdin dimethyl ester crystallised out. M.P. 221° (corr.). It was identical in every respect with glaucobilin ester [Fischer *et al.*, 1932].

The same substance was obtained from II, III and V, but the yield was smaller.

Biliverdin by autoxidation of bilirubin.

0·58 g. of bilirubin was dissolved in 10·5 ml. of 0·22 *N* NaOH and 0·07 ml. of *M*/10 ferrous sulphate solution was added. After 6 days' standing at 30° some precipitated bilirubin (10 mg.) was filtered off and the biliverdin taken up in ether and precipitated therefrom as described below. 150 mg. of crude biliverdin were obtained, which however gave only 10 mg. of well crystallised pure biliverdin

[1] Lindenfeld [1931; 1933] considers the different crystalline forms of haemin and porphyrin esters to be polymorphous modifications.

dimethyl ester. M.P. 220–221° (corr.). The crystals were those termed form A below. No depression of the melting-point was obtained with dehydrobilirubin dimethyl ester [Lemberg, 1932].

The considerable ether-insoluble part of the green pigment was almost insoluble in methanol. From its solution in pyridine it was precipitated by hot methanol in very fine amorphous flocks of almost black colour and resinous character. Dilute methyl alcoholic hydrochloric acid dissolved it with an olive-brown colour due to included bilirubin; more concentrated methyl alcoholic hydrochloric acid again precipitated black flocks.

Mesobiliverdin from the products of the Gmelin test for mesobilirubin.

0·1 g. mesobilirubin was dissolved in 100 ml. of warm chloroform. A few ml. of chloroform were shaken in a test-tube with one drop of fuming nitric acid, and 1 ml. of this added to the mesobilirubin solution. The colour changed slowly to olive-green. After 6 minutes the colour had become green with a yellow tinge at the borders of the liquid. The reaction was stopped at this time by shaking with water containing a little sodium acetate. The chloroform was evaporated and the residue taken up with absolute methanol which left 30 mg. of unchanged mesobilirubin undissolved. The solution was blackish-blue in neutral, blue in acid solution. The acid solution showed, besides the absorption in the red due to mesobiliverdin, a weak band at 590 $m\mu$. On addition of zinc acetate the neutral solution turned green with weak red fluorescence and showed three bands, the strongest at 685 $m\mu$, a weaker one at 630 $m\mu$ and a very weak one at 578 $m\mu$. The violet pigment which causes the band 590 $m\mu$ in acid solution and the bands at 630 and 578 $m\mu$ and the red fluorescence of the zinc salt will be discussed in another paper.

The pigment in the methyl alcoholic solution was esterified and the ester brought into ethereal solution as described above. From the ethereal solution mesobiliverdin ester was extracted with 1 % hydrochloric acid which showed now the characteristic blue-green colour and absorption only in the far red. The by-products, among them the weakly basic violet pigment, remained in the ether. The mesobiliverdin ester was shaken out with fresh ether after addition of dilute sodium carbonate solution. It was recrystallised from methanol. 25 mg. of steel-blue prisms of mesobiliverdin dimethyl ester were obtained. M.P. 220° (uncorr.).

If the Gmelin reaction were allowed to proceed until the blue phase was reached the yield of mesobiliverdin became very small.

Mesobiliverdin by oxidation of mesobilirubinogen with ferric chloride.

0·3 g. mesobilirubinogen (the chromogen of mesobilirubin) was boiled for 1·5 hours with a solution of 1·5 g. $FeCl_3$ in 9 ml. 25 % hydrochloric acid and 50 ml. methanol. On cooling well formed rectangular blue leaflets with straight ends crystallised out. Yield 0·2 g.

Although the crystals looked perfectly uniform, they were mixed crystals of two different substances. Their blue solution in acid alcohol showed, besides strong absorption in the red, a band at 595 $m\mu$ and one at 555 $m\mu$ with a shading between the two. In the neutralised solution on addition of zinc acetate a strong red fluorescence appeared and the blue-green solution showed bands at 685, 631 and 578 $m\mu$. If the crystals were recrystallised twice from methanol containing some HCl flat blue-green needles with straight ends were obtained, whereas the mother-liquor was blue. The solution of the crystals now showed absorption only in the red; the zinc compound was green and without fluorescence. The

mother-liquors gave a blue zinc compound with strong red fluorescence and the bands 631 and 578 $m\mu$; the acid solution showed the bands at 595 and 555 $m\mu$. These bands are caused by mesobiliviolin. The crystals are the ferric chloride double salt of mesobiliverdin dimethyl ester (the "ferrobilin ester" of Fischer *et al.* [1932]). M.P. 276° (not corr.). (Found: C, 52·04; H, 5·34; Cl, 16·81; Fe, 5·72; OCH_3, 7·52 %. $C_{35}H_{42}O_6N_4.HCl.FeCl_3$ requires C, 51·66; H, 5·33; Cl, 17·45; Fe, 6·87; $2OCH_3$, 7·63 %.) The low value for iron is explained by the fact that $FeCl_3$ is volatile and iron was assayed as combustion residue.

Optical properties of the crystals: pleochroism: blue, if long axis of platelets parallel to plane of polarisation (NS), blue-green in perpendicular orientation (EW)[1]. Extinction almost straight.

By mild treatment with alkali they were transformed into mesobiliverdin ester as described by Fischer *et al.* [1932].

Similar mixed crystals to those mentioned above, but richer in mesobiliviolin chloride, result from shorter treatment of mesobilirubinogen with ferric chloride. They have been obtained previously and considered to be pure mesobilicyanin chloride [Lemberg, 1933]. It will be shown in another paper that "bilicyanins" are mixtures of pigments, which are coloured blue-violet in acid, red-violet in neutral solution, with biliverdins. For this reason the term violin is used, although the "mesobiliviolin" of Fischer and Niemann [1924] is a mixture of still more complicated nature.

Preparation and analysis of biliverdin.

Free biliverdin (dehydrobilirubin) is rather insoluble in ether. The yield could be greatly increased and much ether saved by using for extraction ether rich in methyl alcohol.

The crude ferric chloride double salt of biliverdin chloride was prepared from bilirubin by oxidation with ferric chloride in glacial acetic acid as described by Lemberg [1932]. 0·5 g. of this material is dissolved in 30 ml. of $N/5$ NaOH and filtered from ferric hydroxide; 100 ml. of methanol are added, the solution is acidified with dilute acetic acid and immediately poured into 500 ml. ether and shaken. It is then extracted several times with ether, some more methanol being added after each extraction. The solution must be kept acid with acetic acid. The extraction is continued until ether no longer extracts an appreciable amount of the pigment, even if some more methanol has been added. 1·5 l. of ether have been found necessary. The remaining watery solution contains a fine precipitate of ether-insoluble "biliverdin" which is filtered off and dried (80 mg. of this by-product).

The ether solution is washed with water. The first washings remove with the methanol a part of the pigment. This is precipitated by addition of some more sodium acetate (the reaction must remain slightly acid) and filtered off or taken back into ether. The greater part of the biliverdin precipitates from the ether solution when the methanol is washed away with water. If the ethereal filtrate is concentrated to 50 ml. and washed again, a second portion of the pigment is obtained. The ether remains violet and contains some biliviolin.

The green precipitates of the ether-soluble part are collected, washed carefully and dried in the desiccator. They are dissolved by boiling with 200 ml. of methanol. The solution is filtered and concentrated to 30 ml.; 240 mg. of

[1] The phrases North-South (NS) and East-West (EW) refer to the position in the field of view of the polarising microscope.

biliverdin crystallise out. This yield, however, can be obtained from the first crystallisation of the ferric chloride double salt only. A much smaller yield is obtained and more of the amorphous ether-insoluble by-product, if that part of the ferric chloride compound is used which comes out of the glacial acetic acid mother-liquors. Thus the total yield of crystalline product is no more than 30 % of bilirubin.

The substance was dried for analysis at 80° and 0·1 mm. over phosphorus pentoxide. (Found: C, 64·63, 64·89; H, 6·00, 5·95; N, 9·06, 8·91, 9·07 %. $C_{33}H_{34}O_6N_4$ requires: C, 68·01; H, 5·89; N, 9·62. $C_{33}H_{38}O_8N_4$ requires: C, 64·04; H, 6·19; N, 9·06 %.)

The analysis of the dimethyl ester of this preparation showed that it contains six oxygen atoms like that of Lemberg [1932]. The starting material, the crude ferric chloride double salt also contains six oxygen atoms only. (Found: C, 50·73; H, 4·59; N, 6·75, 6·67, 7·04; Cl, 17·54, 17·34; Fe, 6·42, 7·11 %. $C_{33}H_{34}O_6N_4.HCl.FeCl_3$ requires: C, 50·71; H, 4·52; N, 7·17; Cl, 18·18; Fe, 7·16 %.) The first iron value was calculated from the weight of the combustion residue, the second obtained by direct precipitation of ferric hydroxide with ammonia. After the biliverdin had been recrystallised twice from methanol the analysis now confirmed the formula with six oxygen atoms for the free acid as well. (Found: C, 67·87; H, 5·79 %. $C_{33}H_{34}O_6N_4$ requires: C, 68·01; H, 5·89 %.) The analytical values for H show that biliverdin has no more than two hydrogen atoms less than bilirubin. (For the free acid have been found: H, 6·00, 5·95, 5·79 %. $C_{33}H_{34}O_6N_4$ requires: H, 5·89 %; $C_{33}H_{32}O_6N_4$: H, 5·56 %. Dimethyl ester, found: H, 6·42, 6·50, 5·96 % (uteroverdin ester); 6·26 % (oocyan ester); 6·39, 6·44 % (biliverdin ester). $C_{35}H_{38}O_6N_4$ requires: H, 6·28 %; $C_{35}H_{36}O_6N_4$: 5·96 %.)

The ether-insoluble by-product still contains some biliverdin. From its methyl alcoholic solution, methyl alcoholic HCl gives a fine blackish precipitate. If this is filtered off, a mixture of amorphous and crystalline chlorides is obtained by addition of hot 2*N* HCl.

Chlorides of biliverdins.

Mesobiliverdin chloride. 50 mg. of mesobiliverdin are dissolved in 15 ml. of boiling methanol and 40 ml. of hot 2*N* aqueous hydrochloric acid are added. After a short time an amorphous precipitate appears; this is filtered off. The chloride crystallises from the filtrate in fine green needles.

The substance is dried for analysis *in vacuo* at 20° over P_2O_5 to avoid dissociation and loss of HCl. (Found: N, 8·78, 8·98; Cl, 4·46, 4·21 %. $C_{33}H_{38}O_6N_4.HCl$ requires: N, 8·98; Cl, 5·70 %.)

Mesobiliverdin dimethyl ester chloride is obtained in the same way. It crystallises also, on standing, from its solutions in 1 % hydrochloric acid obtained by extracting its ether solution with the former: M.P. 175–176°; no complete melting, but sintering together.

Biliverdin chloride. Fine green needles. Extinction slightly oblique. Pleochroism: (NS) blue-green (darker), (EW) yellowish-green (lighter). (Found: C, 63·38, 63·36; H, 5·52, 5·75; N, 7·02, 6·84; Cl, 4·81, 4·71 %. $C_{33}H_{34}O_6N_4.HCl$ requires: C, 63·99; H, 5·55; N, 9·05; Cl, 5·74 %.) The nitrogen is 2 % too low. On drying at 0·1 mm. and 80° over phosphorus pentoxide for several hours, 9·615 mg. substance lost 0·363 mg., but there was no considerable increase in nitrogen percentage. (Found: N, 7·09 %.) There can be no doubt however about the existence of a monochloride.

Biliverdin dimethylester chloride. Fine green needles with no definite M.P. The crystal properties are given below.

Complex salts of biliverdins.

For these experiments mesobiliverdin ester was used.

Zinc salt. A few ml. of a solution of the ester in methanol were filled into a Thunberg tube, in which was placed a small test-tube containing zinc acetate solution. The tube was evacuated twice and filled with nitrogen free from oxygen. When the solutions were mixed the colour turned immediately from blue to green. The general absorption in the red part of the spectrum gave place to a sharper band, with its centre at 685 $m\mu$. This is the band of the complex zinc salt. There is however no fluorescence.

On addition of water and ether, the zinc compound passed from methanol into the ethereal layer. When this was shaken with $N/10$ HCl, the zinc compound was decomposed; the liberated mesobiliverdin ester passed into the acid, showing the typical green colour.

On standing exposed to the air the zinc compound was slowly changed, much faster if ammonia is added. The red fluorescence and the absorption band at 630 $m\mu$ characteristic of biliviolins appeared.

Copper salt. The formation of the copper salt was carried out in nitrogen as in the case of the Zn salt. The solution turned olive-yellow when the copper acetate solution was added. No distinct band could be seen, but general absorption in the red, blue and violet. When the copper compound was brought into ether and this shaken with $N/10$ HCl, no mesobiliverdin was extracted. The ethereal solution was now green and showed a strong band at 650 $m\mu$. When now $2N$ HCl was added, a play of colours developed. Immediately under the green ethereal layer the acid aqueous layer showed a blue zone with a red-violet one below. Gradually the acid solution turned blue-violet, but after some time green flocks of mesobiliverdin chloride precipitated and left the solution red-violet, showing absorption bands at 600 and 550 $m\mu$.

There must exist a copper compound, or otherwise the mesobiliverdin would have been extracted by the dilute acid from ether. The copper complex salts of pyrrole compounds are more stable towards acid than those of zinc. The stronger HCl however splits the compound and sets free the bile pigment which has been changed partly into a biliviolin. Whether this more rapid change of the copper salt is due to an oxidation by bivalent copper or occurs as an effect of the stronger acid on the copper complex is not clear.

If ammonia were added the olive copper compound gave a blue solution showing the typical bands of complex salts of the biliviolin type at 632 and 578 $m\mu$.

Biliverdin, uteroverdin and oocyan dimethyl esters.

In the paper of Lemberg and Barcroft [1932] Bernal reported that oocyan and uteroverdin esters differed in the optical properties of the crystals. I found later a similar difference between the crystals of uteroverdin ester and biliverdin ester obtained from bilirubin with ferric chloride.

Different preparations of natural and synthetic biliverdin esters have been examined. All crystallise in one of the two forms which may be called A and B or they may contain a mixture of both. Form A is that observed by Bernal for oocyan ester, form B that found by him for uteroverdin ester. In Bernal's report the orientation of the pleochroism of both forms was given inversely to that observed now, which was probably due to a mistake.

Pure A was obtained if pure bilirubin was dehydrogenated whether with ferric chloride or with atmospheric oxygen in alkaline solution. Even impure samples of the ester crystallised then in the A-form only. The purest was one

obtained by esterification of pure crystalline biliverdin; its M.P. was 223° (corr.). But samples melting as low as 195° crystallised as A.

Very small amounts of B were obtained from pure bilirubin twice. Once bilirubin was extracted with boiling glacial acetic acid. A very small amount went into solution and was simultaneously dehydrogenated to biliverdin which gave B on esterification. Again a very small sample of B was got from the ethereal mother-liquor of biliverdin, after the chief part of the biliverdin had separated.

B was predominant in uteroverdin ester, some samples of which were almost free from A. The ester of oocyan contained more A and less B. Mixtures of A and B resulted also from biliverdin extracted from ox gallstones by extraction with boiling glacial acetic acid or by dehydrogenation of bilirubin, which had been prepared from the mother-liquors in chloroform by precipitation with alcohol. Here again the melting-point varied from 196° up to 209° (for uteroverdin ester) and to 215° for the product of the glacial acetic acid extract of gallstones. There was no melting-point depression on mixing the different A or B esters or A with B ester.

No change could be observed from B to A or reverse, on recrystallisation. If a mixture of A and B were fractionated by crystallisation from methanol, A crystallised first, B from the mother-liquors.

The mesobiliverdin synthesised from analytical neoxanthobilirubic acid [Fischer *et al.* 1932] is according to Siedel and Fischer [1933] a mixture of the natural (unsymmetrical) mesobiliverdin with two other symmetrical mesobiliverdins with different position in the side-chains. The crystals of the synthetic mesobiliverdin ester were compared with crystals of that obtained from bilirubin ("natural ester").

Natural ester: oblique extinction; pleochroism extraordinarily strong (NS), violet-grey (EW), blue; at 45°, red-violet.

Synthetic ester: Extinction changing from almost straight to oblique. Pleochroism different in different crystals, partly similar to the "natural" product, partly much less distinct.

Although these facts seem to favour the assumption that A and B forms are structurally different, the exact investigation of the optical properties of the crystals showed that the differences can be explained satisfactorily as due to growth and orientation of the crystals; a particular impurity may cause the substance to crystallise in the B form.

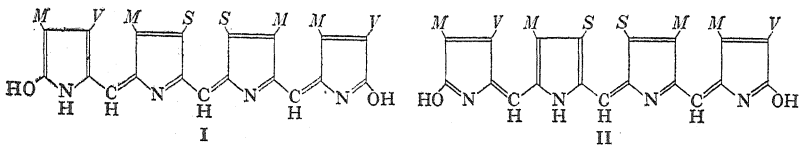
If the crystals are monoclinic, then A may be a form in which the crystals are tabular parallel to (110) or another vertical face elongated along *c*. The extinction would then be oblique. Tilting the crystal along its long axis into the direction in which the light is perpendicular to (100) should give straight extinction, turning it in the opposite direction should increase the angle of extinction up to the value it shows for its (010) plane. That is what actually happens if the A-crystals are tilted on a hemisphere.

The B-crystal may be one showing its (100) or (001) plane. The different pleochroism can be explained by the fact that it is elongated along *b*. Thus on turning the extinction remains straight.

Further evidence against the assumption of a genuine difference between A and B arises from the investigation of the hydrochloride crystals. No difference could be found between the chlorides of pure A-ester and of uteroverdin ester. Both show the following properties: flat needle-shaped green prisms; complete extinction almost straight (deviation less than 2°). Pleochroism: (NS) blue-green (dark in sodium light), (EW) green (lighter in sodium light), slow wave parallel to long axis. The partial birefringence can be roughly estimated to be 0·02.

Discussion.

Two formulae have been discussed for biliverdin [Fischer and Adler, 1931; Lemberg, 1932; Siedel and Fischer, 1933; Lemberg and Bader, 1933] which differ in the position of the double bonds in the rings I and II:



I had preferred formula I in 1932, as biliverdin was then thought to be incapable of forming complex salts, and this formula accounted better for their non-existence. But as complex salts have now been proved to exist, there is no longer any reason for preferring it. Furthermore a substance of formula I should form dichlorides (as the porphyrins do) on its two middle basic nitrogen atoms, whereas in formula II a monochloride, which has been found, is understandable; the two nitrogen atoms of the rings I and IV are less basic being lactim-nitrogen (—N=C(OH)—).

Mesobiliverdin was considered by Siedel and Fischer [1933] to be the blue stage of the Gmelin test. It is shown in this paper that this pigment is the chief product in the green phase of the Gmelin test, whereas it is hardly present when the blue phase is reached. The Gmelin test was carried out at high dilution which favours the successive formation of the single reaction phases. Nevertheless mixtures were obtained. They were separated by use of the Willstätter HCl-number method, which is as applicable to certain types of bile pigments as to porphyrins or chlorophyll derivatives.

Mesobiliverdin has been obtained in the form of the ferric chloride double salt of its ester chloride by oxidation of mesobilirubinogen with ferric chloride. The bearing of this observation on the relation between the green and violet bile pigments (mesobiliviolin and the phycobilins of red algae) and on the urobilin problem will be discussed in a forthcoming paper. A short report has been already given [Lemberg, 1934].

Summary.

The preparation is described of dehydromesobilirubin dimethyl ester from crude "mesobiliverdin" and of dehydrobilirubin ester from "biliverdin." It is proposed that the names biliverdin and mesobiliverdin should be used for the green dehydrobilirubins.

Mesobiliverdin forms the first (green) stage of the Gmelin reaction, from which it could be isolated in a yield of 36 % of the mesobilirubin which entered the reaction.

Mesobiliverdin is obtained from mesobilirubinogen by oxidation with ferric chloride in the form of the ferric chloride double salt of its dimethyl ester chloride ($C_{35}H_{42}O_6N_4.HCl.FeCl_3$).

The method of preparation of biliverdin is improved and its formula $C_{33}H_{34}O_6N_4$ (with two hydrogen atoms less than bilirubin) is confirmed.

Biliverdin dimethyl ester can crystallise in two different forms. The first form is obtained from pure bilirubin. Natural biliverdins (uteroverdin and oocyan) give a second form mixed with more or less of the first; so does biliverdin from the mother-liquors of bilirubin. The two types can be explained by differences of crystal growth and the crystallographic investigation gives no evidence

of the existence of two structurally different substances. Biliverdin, uteroverdin and oocyan may be considered as identical.

Crystalline monochlorides are obtained from biliverdin, mesobiliverdin and their esters. The biliverdins form complex salts with Zn and Cu, but the Zn salt lacks the typical fluorescence of pyrrole pigment zinc complexes. These observations allow of a decision between the two formulae which have been proposed for biliverdin.

REFERENCES.

Fischer and Adler (1931). *Z. physiol. Chem.* **200**, 209.
—— Baumgartner and Hess (1932). *Z. physiol. Chem.* **206**, 201.
—— and Hess (1931). *Z. physiol. Chem.* **194**, 193.
—— and Niemann (1924). *Z. physiol. Chem.* **137**, 293.
—— Treibs and Zeile (1931). *Z. physiol. Chem.* **193**, 138.
Hamsik (1931). *Z. physiol. Chem.* **196**, 195.
Kuhn and Drumm (1932). *Ber. deutsch. chem. Ges.* **65**, 1458.
Lemberg (1931). *Liebig's Ann.* **488**, 74.
—— (1932). *Liebig's Ann.* **499**, 25.
—— (1934). *Chem. Ind.* **53**, 179.
—— and Bader (1933). *Liebig's Ann.* **505**, 151.
—— and Barcroft (1932). *Proc. Roy. Soc. Lond.* B **110**, 362.
Lindenfeld (1931). *Rocznicki Chem.* **11**, 532.
—— (1933). *Rocznicki Chem.* **13**, 645, 660.
Richter (1930). *Z. physiol. Chem.* **190**, 21.
Siedel and Fischer (1933). *Z. physiol. Chem.* **214**, 145.

CXXXVIII. A COLORIMETRIC METHOD FOR THE DETERMINATION OF *N*-ACETYLGLUCOSAMINE AND *N*-ACETYLCHONDROSAMINE.

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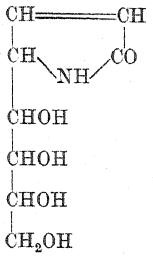
The reaction of acetylated glucosamine with *p*-dimethylaminobenzaldehyde was first observed by Müller [1901] who showed that after warming a solution of penta-acetylglucosamine with dilute potassium hydroxide an intense reddish-purple colour was produced by the addition of a solution of *p*-dimethylaminobenzaldehyde acidified with hydrochloric acid [Ehrlich's reagent; 1901].

A colorimetric method for the quantitative estimation of *N*-acetylglucosamine, based on the above reaction, has recently been described by Zuckerkandl and Messiner-Klebermass [1931]. These workers suggested that when *N*-acetylglucosamine (II) is warmed with dilute alkali the aldehyde group and the acetyl group react together with the subsequent formation of a pyrrole derivative (I) which then condenses with *p*-dimethylaminobenzaldehyde to yield the characteristic red coloration which pyrroles are known to give with Ehrlich's reagent. We have obtained evidence [Elson and Morgan, 1933, 1, 2, and unpublished results], however, that *N*-acetylglucosamine reacts in the enolic form (III) and passes over with loss of water into the oxazole derivative, 2-methyl-4-$\alpha\beta\gamma\delta$-tetrahydroxy-*n*-butyloxazole (IV), and we consider that it is the condensation of this substance with *p*-dimethylaminobenzaldehyde in acid solution which gives rise to the intense reddish purple coloration. The action of dilute alkali on *N*-acetylglucosamine will be considered in more detail in a later communication.



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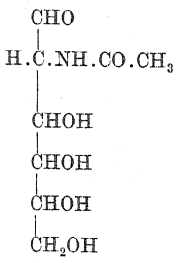

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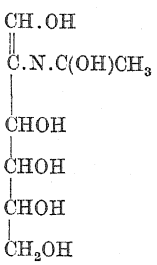
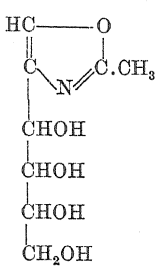


III



IV

According to the procedure of Zuckerkandl and Messiner-Klebermass *N*-acetylglucosamine (1–4 mg.), after dissolution in 5 ml. of 60 % aqueous alcohol, is heated for 6–8 seconds at boiling-point with two drops of 30 % potassium hydroxide solution and rapidly cooled. The *p*-dimethylaminobenzaldehyde reagent (1 ml.) is then added and after 10–15 minutes the reddish-

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purple colour which develops is matched colorimetrically against the colour produced by a standard solution of *N*-acetylglucosamine which has been treated in the same manner.

In our experience this method cannot be used where accurate and reproducible results are required. It is not proposed to discuss here the numerous variations in technique which have been tried in order to render the method suitable for our requirements, but only to state that investigations were carried out to determine the optimum conditions under which *N*-acetylglucosamine could be converted into the oxazole derivative (IV) and to ascertain the factors which are necessary for the production of the maximum colour intensity per mg. of the oxazole derivative when this compound is condensed with *p*-dimethylaminobenzaldehyde.

Early in our work it was found that unless the operation of boiling the acetylglucosamine in alkaline alcoholic solution were very carefully controlled a considerable proportion remained unconverted into the oxazole derivative or was destroyed by the action of the alkali. In view of the marked influence of potassium hydroxide upon the quantitative conversion of the *N*-acetylhexosamine into the corresponding oxazole under these conditions we consider that—"two drops of 30 % potassium hydroxide"—and—"heating for 6–8 seconds at boiling-point"—as recommended by Zuckerkandl and Messiner-Klebermass, do not define the concentration of alkali present in the test solution, or the time of heating, with sufficient accuracy for a quantitative procedure.

A greater colour intensity per mg. of acetylglucosamine results when the colour reaction is carried out in 90 % acetic acid solution and by the use of this solvent it has been possible to estimate quantities of acetylglucosamine as small as 0·08 mg. A perceptible colour is obtained with 0·01 mg. acetylglucosamine in a volume of 10 ml.; the test is therefore sensitive at a dilution of 1 in a million of acetylglucosamine.

Experimental.

Reagents.

(1) p-*Dimethylaminobenzaldehyde reagent.* *p*-Dimethylaminobenzaldehyde (A. R.) (2 g.) after two crystallisations from dilute acetic acid is dissolved in 100 ml. of glacial acetic acid containing 5·0 ml. of hydrochloric acid (A. R.). The final solution should possess a pale yellow colour. The addition of 1 ml. of water to 9 ml. of the reagent should not produce an increase in the intensity of the yellow colour. Some preparations of *p*-dimethylaminobenzaldehyde (A. R.) have been found to give yellow-coloured acetic-hydrochloric acid solutions which produce bright yellow solutions on the addition of water; these must be avoided. The reagent keeps indefinitely.

(2) N-*Acetylglucosamine standard.* *N*-Acetylglucosamine (1 g.) is dissolved in about 10 ml. of water saturated with chloroform and made up to a volume of 100 ml. From this standard solution dilutions which will contain a required amount of substance per ml. can readily be made. The standard solution should be kept at 0°.

(3) *0·5* N *sodium carbonate solution.*

Procedure.

A solution of *N*-acetylglucosamine, containing 0·1–1·0 mg. per ml., is measured into test-tubes of approximately equal thickness graduated at 10 ml.; if the volume of solution taken is less than 1·0 ml. it should be made up to 1·0 ml. by the addition of water. The sodium carbonate (0·1 ml.) is added and

the tubes are heated in a boiling water-bath for 5 minutes and then cooled. Standard solutions of *N*-acetylglucosamine are treated in the same manner. Glacial acetic acid is run into the tubes from a burette or separating funnel until it is within about 2 ml. of the 10 ml. mark. The *p*-dimethylaminobenzaldehyde reagent (1 ml.) is then added and the solution is made up to 10 ml. with acetic acid, thoroughly mixed and allowed to stand. The colour develops quickly and reaches its maximum intensity in about 45 minutes. When the maximum colour reached is compared with a stable artificial colour standard it shows no appreciable fading over a period of at least 1 hour.

As with most colorimetric methods, this method gives good results only when the colour intensities of the standard and the unknown solutions are approximately the same. Table I shows the results obtained by using the above

Table I.

N-Acetylglucosamine				*N*-Acetylglucosamine			
Standard mg.	Present mg.	Found mg.	% error	Standard mg.	Present mg.	Found mg.	% error
0·90	1·00	1·00	0·0	0·400	0·350	0·355	+1·4
,,	0·95	0·96	+1·0	,,	,,	0·350	0·0
,,	,,	0·97	+2·1	0·350	0·400	0·405	+1·2
,,	0·85	0·84	−1·2	,,	0·300	0·310	+3·3
,,	,,	0·86	+1·2	0·325	0·350	0·345	−1·4
,,	,,	,,	+1·2	,,	0·300	0·305	+1·7
,,	0·80	0·81	+1·3	0·300	0·275	0·275	0·0
0·80	0·90	0·89	−1·1	,,	0·250	0·255	+2·0
,,	0·70	0·70	0·0	0·225	,,	,,	+2·0
0·70	0·80	0·78	−2·5	0·175	0·200	0·205	+2·5
,,	,,	0·79	−1·2	0·150	0·175	0·174	−0·6
,,	0·65	0·64	−1·5	,,	,,	0·177	+0·6
,,	0·60	0·62	+3·3	0·090	0·100	0·102	+2·0
,,	,,	0·61	+1·6	,,	,,	0·099	−1·0
0·55	,,	0·60	0·0	,,	,,	0·101	+1·0
,,	,,	0·585	−2·5	,,	0·080	0·078	−2·5
,,	0·50	0·510	+2·0	,,	,,	,,	−2·5
,,	,,	0·500	0·0	,,	,,	0·079	−1·2
0·450	,,	0·510	+2·0				
,,	,,	0·495	−1·0				
,,	0·40	0·395	−1·2				

procedure when the solutions to be estimated differ in their *N*-acetylglucosamine content by not more than 20 % from that of the standard solution. The method is equally applicable to the estimation of *N*-acetylchondrosamine and it has been

Table II.

N-Acetylchondrosamine			
Standard mg.	Present mg.	Found mg.	% error
0·90	1·00	1·02	+2·0
,,	,,	1·00	0·0
,,	0·85	0·84	−1·2
,,	,,	0·86	+1·2
0·60	0·65	0·65	0·0
,,	,,	0·64	−1·5
,,	0·55	0·56	+1·8
0·550	0·500	0·505	+1·0
0·400	0·450	0·445	−1·1
,,	0·350	0·365	+4·3
,,	,,	0·360	+2·9
0·090	0·100	0·101	+1·0
,,	,,	0·102	+2·0

found that equal amounts of *N*-acetylglucosamine and *N*-acetylchondrosamine when compared under similar conditions give rise to colours identical in tint and intensity. A few results of estimations carried out with *N*-acetylchondrosamine solutions are given in Table II.

A comparison between the results obtained with the original method of Zuckerkandl and Messiner-Klebermass and those obtained using the new technique is shown in Table III. In each series of determinations the results of

Table III.

Experiment no.	Zuckerkandl and Messiner-Klebermass's method 2·00 mg. acetylglucosamine		New technique 0·500 mg. acetylglucosamine	
	Found mg.	% error	Found mg.	% error
1	2·12	+6·0	0·502	+0·4
2	2·16	+8·0	0·498	−0·4
3	1·95	−2·5	0·500	0·0
4	1·95	−2·5	0·498	−0·4
5	2·10	+5·0	0·492	−1·6
6	1·98	−1·0	0·502	+0·4
7	2·18	+9·0	0·500	0·0
8	1·94	−3·0	0·500	0·0
9	1·91	−4·5	0·490	−2·0
10	2·03	+1·5	0·502	+0·4
11	2·00	0·0	0·505	+1·0
12	2·08	+4·0	0·500	0·0

twelve consecutive estimations are given. It will be seen that with the old method the variation between the individual results is of the same order as that observed by Zuckerkandl and Messiner-Klebermass and may exceed 5 %. The results obtained with the modified technique show a variation of only 1–2 % from the true value.

Table IV. *The influence of foreign substances upon the accuracy of the estimation.*

Substance	Added mg.	*N*-Acetylglucosamine hydrochloride: Present mg.	Found mg.	% error
Glucose	1·0	0·500	0·500	0·0
,,	,,	,,	0·505	+1·0
,,	2·5	0·250	0·245	−2·5
Arabinose	1·00	0·500	0·500	0·0
,,	,,	,,	0·500	0·0
Fructose	1·00	0·500	0·500	0·0
,,	,,	,,	0·505	+1·0
,,	2·50	0·250	0·240	−4·0
Alanine	1·00	0·500	0·510	+2·0
,,	,,	,,	0·500	0·0
Histidine	,,	,,	0·495	−1·0
,,	,,	,,	0·510	+2·0
Glucosamine hydrochloride	,,	,,	0·505	+1·0
,,	,,	,,	0·500	0·0
1-Aminoglucose	,,	,,	0·500	0·0
,,	,,	,,	0·510	+2·0
N-Acetyl-1-aminoglucose	,,	,,	0·500	0·0
,,	,,	,,	0·505	+1·0

The influence of foreign substances.

Various sugars and amino-acids have been added to known amounts of *N*-acetylglucosamine in order to ascertain whether the presence of these substances has any influence upon the accuracy of the estimation. A few results of these tests are given in Table IV from which it will be seen that none of the substances added interfered with the determination.

Certain pyrrole and indole derivatives develop coloured solutions when treated with the *p*-dimethylaminobenzaldehyde reagent, and their presence in the solutions to be estimated will therefore influence the determination. Tryptophan does not appear to react with *p*-dimethylaminobenzaldehyde and does not yield a coloured solution under the conditions described, but the presence of moderate amounts (0·1–1·0 mg.) of this amino-acid in the *N*-acetylglucosamine solutions has been found to cause some delay in the rate of development of the reddish purple colour with resulting underestimation of the true result.

The influence of the time of heating and of the concentration of alkali on the subsequent colour development.

Estimations were made on solutions containing 0·5 mg. of *N*-acetylglucosamine per ml. The concentration of the alkali varied from 0·02 *N* to 2 *N* and three or four different periods of heating were tested with each alkali concentration. It is not necessary to give here the results obtained with all of these modifications but only to state that with concentrations of alkali higher than 0·5 *N* the solution, after heating at 100° for more than 2 minutes, developed a final colour intensity which indicated that a considerable destruction of either the *N*-acetylglucosamine or the corresponding oxazole derivative had taken place. The results obtained using 0·02 *N* and 0·1 *N* alkali are shown in Table V. It will be seen that the greatest colour intensity is attained when the *N*-acetylglucosamine

Table V. *Influence of the time of heating with various concentrations of alkali on the subsequent colour development.*

Alkali used	Concentration of alkali *N*	Time of heating min.	Colorimeter reading at maximum colour developed mm.
Na_2CO_3	0·02	5	9·0
,,	,,	10	8·8
,,	,,	15	8·9
,,	0·10	2	11·5
,,	,,	3	9·3
,,	,,	4	9·1
,,	,,	5	9·0
,,	,,	6	9·3
,,	,,	10	13·2
,,	,,	15	18·9
KOH	0·02	2	12·5
,,	,,	3	10·1
,,	,,	4	10·6
,,	,,	5	11·4
,,	0·10	2	10·2
,,	,,	3	13·2
,,	,,	4	20·6
Na_2HPO_4	0·10	5	21·6
,,	,,	10	17·2
,,	,,	30	14·8
,,	,,	40	13·2

is heated for 10 minutes at 100° in 0·02 *N* Na_2CO_3. If 0·02 *N* KOH is used in place of the 0·02 *N* Na_2CO_3 the maximum colour intensity which is reached for the same amount of *N*-acetylglucosamine is somewhat less. This is due to the action of the potassium hydroxide on the oxazole derivative, and the exact extent to which decomposition proceeds can readily be ascertained if a standard solution of the pure oxazole (IV) is treated with alkali under similar conditions.

The production of the oxazole derivative by heating with 0·02 *N* Na_2CO_3 appears to be almost quantitative. To show this a solution of maximum colour intensity which has been derived from a known amount of *N*-acetylglucosamine is compared colorimetrically with the colour developed by a solution of the pure oxazole which has been similarly treated with the *p*-dimethylaminobenzaldehyde reagent. Theoretically 0·5 mg. of *N*-acetylglucosamine should yield 0·459 mg. of the oxazole derivative. After treatment with 0·02 *N* Na_2CO_3 as described above, 0·5 mg. of *N*-acetylglucosamine gave a solution the colour intensity of which was equal to the colour given by 0·440 mg. of the oxazole; the conversion was therefore 96 % complete.

The production of a closed ring structure by the action of very low concentrations of alkali suggested that the reaction would probably take place at an alkalinity which could be considered to be within the range necessary for normal biological processes. The results obtained when *N*-acetylglucosamine was heated with 0·1 *N* Na_2HPO_4 (p_H 8·4) are shown in Table V. It will be seen that approximately 65 % of the *N*-acetylglucosamine has been converted into the oxazole derivative after 40 minutes' heating at 100°. A sterile solution of *N*-acetylglucosamine which has been buffered at p_H 8·4 with sodium phosphate and kept at 37° showed after 48 hours that about 10 % had been converted into the oxazole.

Although the maximum colour intensity reached per mg. of *N*-acetylglucosamine is slightly less when 0·1 *N* Na_2CO_3 is used in place of 0·02 *N*, we consider that for practical purposes a higher concentration of alkali than 0·02 *N* is more reliable since it is then possible to neglect slight traces of acid when these are present in the solution to be estimated.

The influence of the concentration of the hydrochloric acid upon the colour intensity.

A series of eight test-tubes containing *N*-acetylglucosamine (0·5 mg.) dissolved in 1·0 ml. of water was heated with 0·1 ml. of 0·5 *N* Na_2CO_3 for 5 minutes in a boiling water-bath and cooled. The tubes were then treated one at a time in the following manner. About 7·0 ml. of glacial acetic acid were added to each together with 0·1 ml. 0·5 *N* HCl and 1·0 ml. of *p*-dimethylaminobenzaldehyde (2 %) dissolved in glacial acetic acid which contained in the different experiments concentrations of hydrochloric acid[1] varying from 1 to 40 %. The tubes, after filling to the 10 ml. mark with acetic acid, were thoroughly shaken and the intensity of the colour produced was followed in each case by means of colorimetric observations at prearranged intervals against a stable artificial colour standard for a period of 90 minutes. The results are shown graphically in Fig. 1. It is seen that a reduction in the concentration of hydrochloric acid from 40 to 2·5 % in the *p*-dimethylaminobenzaldehyde reagent causes a steady increase in the intensity of the maximum colour produced; the rate of colour development is however slower with the lower acid concentrations and the colour formed is considerably more stable. When the reagent contains 20 or 30 % HCl, the

[1] Expressed throughout as % of concentrated HCl A. R. present.

intensity of the reddish purple colour reaches its maximum in less than 10 minutes and then rapidly fades. A concentration of hydrochloric acid of 5 % in the reagent causes the colour intensity to develop for about 45 minutes and then remain practically constant for a further 60 minutes. The colour development when 2·5 % of hydrochloric acid is employed is very slow, and the intensity of the colour developed does not reach its maximum until after about 90 minutes have elapsed; 1·0 % of acid in the reagent produces only a trace of colour after 2 hours. It should be emphasised however that the rate of colour development increases rapidly with increasing temperature and that the curves shown were obtained within the temperature range 13–16°.

From these observations it appears that the colour is very sensitive to an excess of hydrochloric acid and that, with concentrations of acid exceeding 0·5 % in the final acetic acid solution, fading of the colour goes on concurrently with colour production.

A similar series of curves is obtained when standard solutions of the pure oxazole derivative (IV) are treated in the same manner with the *p*-dimethylaminobenzaldehyde reagent containing different concentrations of hydrochloric acid.

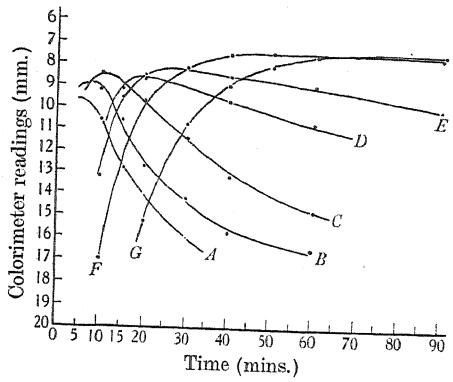

Fig. 1.

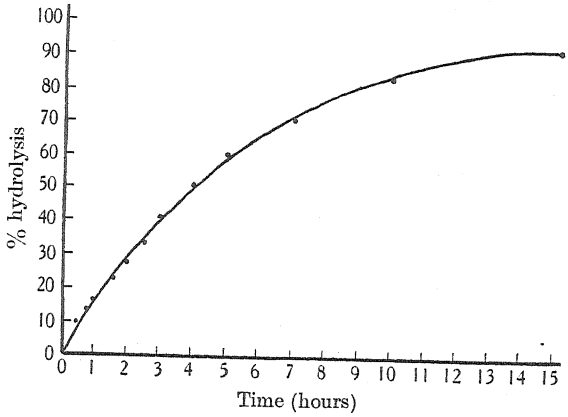

Fig. 2.

Fig. 1. The influence of the concentration of hydrochloric acid on the rate and intensity of colour development. Curve *A* 40 %, *B* 30 %, *C* 20 %, *D* 15 %, *E* 10 %, *F* 5 %, *G* 2·5 % of hydrochloric acid in the *p*-dimethylaminobenzaldehyde reagent.

Fig. 2. Curve showing rate of hydrolysis of *N*-acetylglucosamine with 0·1 *N* sulphuric acid.

Application.

The method has been used to follow the rate of hydrolysis of the acetyl group in *N*-acetylglucosamine, and the results of a typical hydrolysis experiment are shown in Fig. 2. The final figure shows that 94 % of the acetyl groups had been eliminated in 15 hours, and this figure was confirmed by estimation of the glucosamine formed using the colorimetric method described by Elson and Morgan [1933, 1]. Similarly the rate of esterification of *N*-acetylglucosamine to yield the non-reducing 1-methyl-*N*-acetylglucosamine and the hydrolysis of this compound back to *N*-acetylglucosamine can be readily followed by the colorimetric method described. The procedure has also been used to determine the rate at which the *N*-acetylhexosamine units, which are contained in the specific polysaccharide of *B. dysenteriae* (Shiga), are liberated by acid or enzymic hydrolysis [Morgan, 1931; 1932; Morgan and Thaysen, 1933].

SUMMARY.

A colorimetric method for the determination of *N*-acetylglucosamine, which was elaborated by Zuckerkandl and Messiner-Klebermass and which is based on a reaction originally described by Müller, has been studied. The procedure recommended by them has been found to give unsatisfactory results. By changing the technique it is possible to obtain reproducible results and at the same time to reduce considerably the amount of substance necessary for the determination. *N*-Acetylchondrosamine can be estimated by the modified method.

REFERENCES.

Ehrlich (1901). *Med. Woch.*
Elson and Morgan (1933, 1). *Biochem. J.* **27**, 1824.
—— —— (1933, 2). *Chem. Ind.* **53**, 844.
Morgan (1931). *Brit. J. Exp. Path.* **12**, 62.
—— (1932). *Brit. J. Exp. Path.* **13**, 342.
—— and Thaysen (1933). *Nature*, **132**, 604.
Müller (1901). *Z. Biol.* **42**, 564.
Zuckerkandl and Messiner-Klebermass (1931). *Biochem. Z.* **236**, 19.

CXXXIX. ON THE NATURE OF THE PRECURSOR OF THE VITAMIN C IN THE VEGETABLE KINGDOM.

I. VITAMIN C IN THE GROWING PEA SEEDLING.

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In an earlier paper [Harris and Ray, 1933], it was shown that ascorbic acid appears in pea seeds as soon as germination begins. Since then the rate of production of ascorbic acid in the seedlings of such pea seeds has been examined, and the result is embodied in Table I. For the estimation of the acid, the

Table I.

Period of germination	Wt. of 20 seedlings g.	Amount of ascorbic acid mg./g. seedlings
12 hours (seeds kept soaked in water)	0·18	0·00
48 hours (germinated)	0·29	0·23
60 ,, ,,	0·60	0·43
90 ,, ,,	1·70	0·50
115 ,, ,,	2·60	0·50
140 ,, ,,	2·50	0·48
170 ,, ,,	4·2	0·49
12 days ,,	4·8	0·49

micro-chemical method of Birch *et al.* [1933] was used. Yellow-pea seeds were kept in sand slightly moistened with water, and after various periods of germination were taken out and washed with water to remove the adhering sand, and the cotyledons were excised away. The separated portions (here termed the seedlings) were dried between two filter-papers and ground up with a little sand and sufficient 20 % trichloroacetic acid to give a final concentration of the latter of about 5 %. The extracts were made up to a suitable volume and filtered, and the filtrates were titrated against a standard solution of 2 : 6-dichlorophenolindophenol. As a rule, 20 seedlings were used for each estimation.

As will be noticed, the amount of ascorbic acid present per g. of the wet seedling tissue increases within a few hours from 0 to a constant value which is not affected by any further growth. This is represented graphically in Fig. 1.

It may, however, be remarked that though the young seedlings are quite rich in vitamin C, the whole of the vitamin content is not confined to them. Judged from separate titration results on the seedlings and the cotyledons, the latter have been found to account for more than 5/6 of the total reducing power. It is doubtful, however, whether the whole of the reducing power of the cotyledons is due to ascorbic acid. It is known that a few substances like cysteine reduce the dye under the conditions of the experiment. As extracts made from the cotyledons show a marked nitroprusside reaction, and as Van Eekelen *et al.* [1934] have

shown that part of the reducing material in the cotyledons is precipitated by mercuric acetate, it seems probable that some free cysteine may be present in them. Johnson [1933] has also found that extracts from germinating peas show much less antiscorbutic value than was to be expected from the titration results. On the other hand extracts from seedlings alone show no nitroprusside reaction,

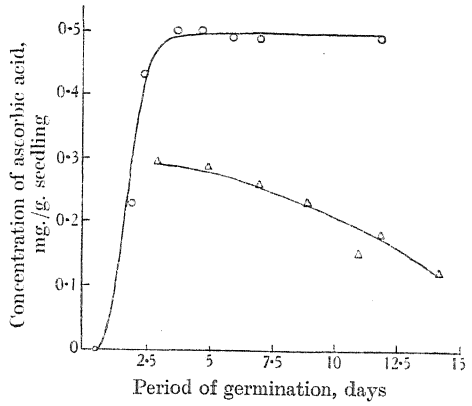

Fig. 1. Curve showing rate of production of ascorbic acid during various periods of germination or cultivation.

o, seedlings grown naturally with the cotyledons.
Δ, seedlings grown artificially on a medium containing fructose.

and they reduce the indicator solutions as rapidly as those of pure ascorbic acid solutions. These findings confirm those of Johnson, who also believes that the reducing power of seedlings is due to ascorbic acid alone.

Nature of the precursor of vitamin C in plants.

Though the synthesis of vitamin C during germination has been known for a long time, no attempt seems to have been made to ascertain the nature of the substance or substances from which it is formed. In their classical work Brown and Morris [1890] had shown that embryos of barley, excised out of the endosperm and cultivated upon nutrient solution, grew steadily at the expense of the carbohydrates or other organic nutrient substances present in the solution. It was thought that by utilising this method, *i.e.* by growing the embryo seedlings of peas on nutrient solutions containing various organic compounds and examining in the different samples whether ascorbic acid is formed or not, some information about the nature of the precursor might be obtained. Experiments in these directions have been successful and some very interesting results have been obtained. These will be discussed below.

Experimental.

The mode of experiment usually adopted was to place the excised embryo seedlings on a suitable semi-solid medium. It is essential that the embryo seedlings should rest on the medium and not be immersed in it, as in the absence of air growth and production of ascorbic acid do not take place.

It was found that a 10 % gelatin mixture constitutes an ideal medium for the cultivation of the seedlings. Gelatin itself is not assimilable by the young

plant and the 10 % solution forms a suitable semi-solid gel, whose viscosity is sufficient to prevent the embryo seedlings from sinking in it, but not great enough to stop the radicles from piercing it and absorbing the proper nutrient substances. In order to provide sufficient nitrogen and other elements necessary for a growing plant, the gelatin was dissolved in the proper quantities of the following solution (Knopp's solution):

Calcium nitrate	0·8 g.
Potassium nitrate	0·2 g.
Potassium dihydrogen phosphate ...	0·2 g.
$MgSO_4, 7H_2O$	0·2 g.
Ferric phosphate	Trace
Water	1000 ml.

The various organic substances to be tested were dissolved in this solution in a concentration of 5 %.

At first, great difficulty was experienced in keeping the medium free from bacterial contamination. If so infected, the seedlings failed to grow and little or no ascorbic acid could be detected in them. The following procedure was, however, found to give satisfactory results, and in most cases no appearance of bacterial life or signs of fermentation could be seen for the experimental period of 7 days.

1 g. of purified gelatin is dissolved in 10 ml. of the Knopp's solution containing 0·5 g. of the experimental compound. While still hot and liquid, the gelatin mixture is transferred to a 25 ml. wide-mouthed flask, fitted with a cotton-wool stopper. The flask with the mixture is then sterilised by steaming for half an hour on 3 successive days. It is then left aside for the gelatin to set. In certain cases where it was feared that the drastic steaming process might hydrolyse or decompose the experimental organic substance, the latter was not added at first, but after the gelatin mixture (8 ml.) had been sterilised the compound was dissolved in a small amount (2 ml.) of sterilised Knopp's solution and was added to the gelatin mixture before it had set. Such cases are marked in Table II with an asterisk.

Before the embryo seedlings are excised, the pea seeds are soaked in sterile distilled water for 6 hours. The seed-coats are then removed and with a little pressure the two cotyledons can be separated easily and the embryo seedling taken out by means of a pair of forceps with bent heads. The seedlings are then washed with several changes of sterilised distilled water, and finally placed on the solidified nutrient gelatin. These operations of washing and transference should be carried out as far as practicable in a dust-free room.

The seedlings after suitable periods of cultivation (usually 7 days unless otherwise mentioned) are taken out and suspended in warm water (37°) to remove any adhering gelatin. They are then dried between two filter-papers and extracted as usual with sand and trichloroacetic acid. The ascorbic acid in the extracts is estimated by titration against the standard 2 : 6-dichlorophenol-indophenol solution. The results are given in Table II.

From the data (Table II) it may be seen that while little reducing substance is produced in the embryo seedling when kept at about 20° on 10 % nutrient gelatin alone, a considerable amount appears when certain substances are introduced into the medium. Of these, hexoses seem to function best. The relative ascorbic acid-producing power of the individual hexoses seem to be more or less the same, mannose being, however, an exception, the seedlings being able to generate much more ascorbic acid from mannose than from any of the other three hexoses

Table II. *Results after 7 days' cultivation.*

Nature of the medium	Wt. of 20 seedlings	Amount of ascorbic acid, mg./g. wet seedling tissue
10 % gelatin solution alone	0·49	0·049
,, ,, ,,	0·46	0·050
,, ,, +glucose	0·87	0·194
,, ,, ,,	0·40	0·250
,, ,, ,,	0·80	0·225
,, ,, +fructose	0·80	0·300
,, ,, ,,	1·03	0·281
,, ,, ,, *	0·93	0·264
,, ,, +galactose	0·48	0·237
,, ,, ,,	0·85	0·168
,, ,, ,, *	0·55	0·201
,, ,, +mannose	0·37	0·540
,, ,, ,,	0·36	0·691
,, ,, +sucrose	0·98	0·275
,, ,, ,,	0·55	0·320
,, ,, ,, *	0·60	0·310
,, ,, +lactose	0·60	0·130
,, ,, +maltose	1·15	0·208
,, ,, +arabinose	0·63	0·0
,, ,, ,, *	0·50	0·0
,, ,, +xylose	0·74	0·120 (?)
,, ,, ,, *	0·63	0·027
,, ,, ,, *	0·60	0·054
,, ,, +starch	0·57	0·061
,, ,, +dextrin	0·78	0·078
,, ,, +mannitol	0·43	0·039
,, ,, +sorbitol	0·43	0·0
,, ,, +inositol	0·50	0·054
,, ,, +sodium pyruvate	0·15	0·0
,, ,, +methylglyoxal	0·15	0·0
,, ,, +sodium glutamate	0·20	0·0
,, ,, +glycerol	0·92	0·079
,, ,, +sodium lactate	0·18	0·0
,, ,, +sodium glycerophosphate*	0·20	0·0

* Substance added to gelatin afterwards (see p. 998).

examined. Disaccharides come next in the list but here it is possible that ascorbic acid is not derived from the disaccharides themselves but from the products of their enzymic hydrolysis. Polysaccharides seem to yield a small, though definite, amount of ascorbic acid, but here again it is doubtful whether they themselves or secondary products are responsible. Pentoses yield consistently negative results; the high value obtained in one case from xylose is probably due not to xylose but to the decomposition products arising during the drastic process of long steaming. Of various other compounds examined only glycerol seems to have a little power while all the rest either gave absolutely negative values or values equal to that given by control seedlings grown on nutrient gelatin alone.

It seems very probable, therefore, that ascorbic acid may be produced by the growing embryo from the hexoses present in the seeds. On the other hand, the concentration of ascorbic acid in the seedlings germinated on hexose media is rather low (0·2–0·3 mg. as compared with 0·5 in seedlings grown with their natural cotyledons); mannose, however, is an exception, the concentration of ascorbic acid being similar to that found under natural conditions. These rather low values may mean that the real precursor is perhaps a substance different from the hexoses. If such a substance exists, however, it is quite evident that its addition is not essential for the production of ascorbic acid and that embryo seedlings are well equipped with enzyme systems to convert ordinary hexoses into this hypothetical substance. About the course of the conversion of hexoses

into ascorbic acid nothing is known. Attempts to grow the seedlings on media containing some of the well-known carbohydrate breakdown products have failed (see Table II). The presence of these substances in the nutrient gelatin seems to inhibit growth, and no trace of ascorbic acid can be detected in the seedlings. Similarly all efforts to elucidate the nature of the enzyme system responsible for this synthesis have been unsuccessful. Attempts to produce ascorbic acid from sugar solutions by means of minced or crushed embryo seedling tissue have only given negative results.

Another interesting thing to be observed from Table II is that there seems to be no direct relation between extent of growth and production of ascorbic acid. Thus the seedlings which are grown on mannose show very little growth, whereas they are exceptionally rich in ascorbic acid. On the other hand, arabinose, xylose, dextrin, all seem to favour growth, but the production of ascorbic acid in these cases is either nil or very low. Substances like fructose seem to favour both growth and production of ascorbic acid.

Antiscorbutic activity of seedlings grown on nutrient gelatin.

Though the chemical determination of ascorbic acid is fairly specific it has certain limitations and may give high values in certain cases [Birch *et al.*, 1933; Harris, 1933]. In view of this it was thought best to check the chemical estimation against direct feeding tests. The biological determination gave results in complete agreement with the indophenol titration. The seedlings used for this purpose

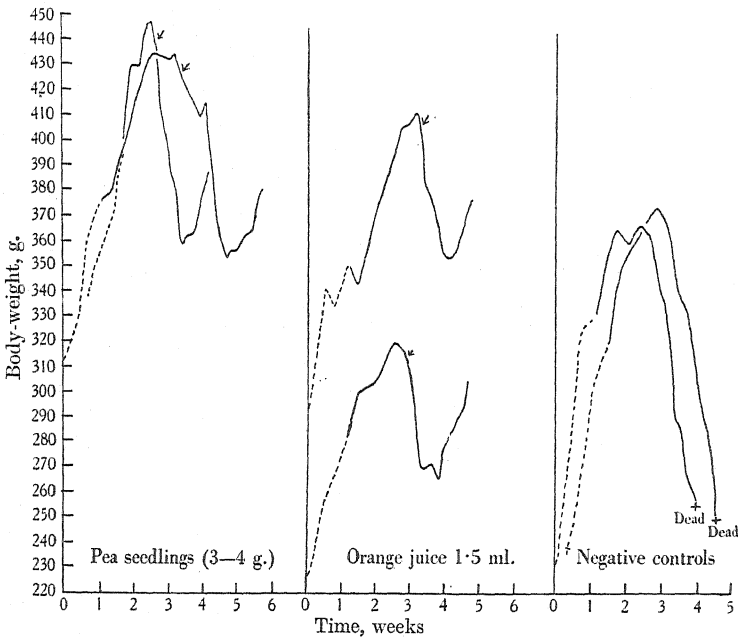

Fig. 2. Weight curves of guinea-pigs, comparing degrees of cure of scurvy with doses of pea seedling and orange-juice.

- - - - -, preliminary complete diet.
———, vitamin C-free diet.
↓, experimental dosing commenced.
+, animal died.

were grown for 7–10 days on nutrient gelatin containing fructose. As it was practicable to grow only a limited amount of these only one level of dosage could be employed. Two guinea-pigs were used for this test and the technique of assay was the curative method described in a previous paper [Harris and Ray, 1932]. A daily dose of 3–4 g. (ascorbic acid content as determined by indophenol titration being 0·75–0·9 mg.) was given to each of the animals. A control group of two guinea-pigs received 1·5 ml. of orange-juice (ascorbic acid content determined chemically about 0·9 mg.) and negative controls were kept on the basal diet alone. The individual growth curves are given in Fig. 2.

3–4 g. of the seedling were found to have nearly the same biological activity as 1·5 ml. of orange-juice. This result corresponds within the limits of experimental error with that calculated from the chemical titration. This shows that the reducing power of the seedling is due solely to the ascorbic acid.

Rate of production of ascorbic acid in seedlings grown on nutrient gelatin containing fructose.

It has already been remarked that using different substances as substrate, no correlation can be detected between growth and synthesis of ascorbic acid. It seemed of interest to examine whether any such correlation exists when the seedlings are cultivated for various periods on media containing one and the same substance. For this purpose, embryo seedlings were grown on media containing fructose (5 %) as the substrate. In order to avoid formation of secondary products during the steaming process, the fructose dissolved in a little sterilised Knopp's solution was added to the gelatin mixture after the latter had been steamed. A large number of flasks each containing 25 embryo seedlings were used and after the proper time of cultivation these were opened, 20 of the best seedlings being taken out for every day's estimation. The result is given in Table III.

Table III.

Period of cultivation days	Wt. of 20 seedlings g.	Total amount of ascorbic acid, mg. in the 20 seedlings	Ascorbic acid, mg./g. seedling
3	0·58	0·170	0·293
5	0·71	0·200	0·282
	0·63	0·180	0·285
7	0·93	0·246	0·264
9	1·35	0·318	0·235
11	1·10	0·165 (?)	0·150 (?)
12	1·20	0·216	0·180
14	1·40	0·177	0·127

It will be noticed that as a rule the greater the growth, the less was the concentration of the ascorbic acid formed, but no simple relation between the two appeared to exist; on the other hand, the concentrations when plotted against the periods of cultivation were found to form a continuous curve (see Fig. 1). The shape of the curve shows that ascorbic acid was produced in great quantities during the first 3 or 4 days of cultivation; afterwards however the rate of formation fell and became zero after about 10 days. After that period some of the ascorbic acid seemed to disappear. On the other hand, the rate of growth continued unchanged and as a consequence the concentration steadily fell. This behaviour is entirely different from that seen in seedlings grown with their cotyledons, the concentration of ascorbic acid rises in these conditions to a constant value.

Correlation between concentration of substrate and production of ascorbic acid.

A further lack of correlation between growth and the synthesis of ascorbic acid was observed when the production of ascorbic acid in seedlings grown for 7 days on media containing varied amounts of fructose was measured (Table IV).

Table IV.

Percentage concentration of sugar solution	Wt. of 20 seedlings	Total amount of ascorbic acid, mg. in the 20 seedlings	Ascorbic acid, mg./g. seedling
1·0	1·2	0·100	0·083
2·5	1·4	0·217	0·155
5·0	0·93	0·246	0·264
7·5	0·80	0·210	0·263
10·0	0·60	0·156	0·260

It is at once apparent that the rate of growth and the rate of production of ascorbic acid follow two different lines, the optimum concentration of the substrate for growth being about 2·5 %, while for the production of ascorbic acid it is much higher, about 5 %. Further the two rates plotted against the concentration form two different types of curves emphasising thereby that the two phenomena are quite different and possibly independent of each other (see Fig. 3).

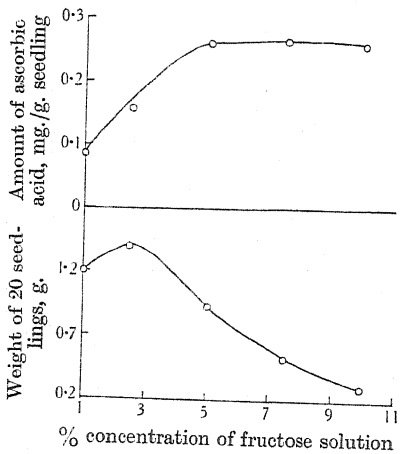

Fig. 3. Relation of rate of production of ascorbic acid by pea seedlings (upper curve) and rate of growth (lower curve) to the concentration of the substrate in the medium on which the seedlings are grown.

Cultivation of seedlings on media containing a mixture of substances.

From Table II it was seen that while some substances favour growth, others seem conducive to the production of ascorbic acid. Attempts were made to combine the two properties, *i.e.* to have good growth together with large production of ascorbic acid. For this embryo seedlings were grown for 7 days on media containing a mixture of two substances—the one favouring growth and the other production of ascorbic acid. As however the results in Table V show, these experiments have not been successful. The volume of medium was 10 ml.

Table V.

Nature of medium	Wt. of 20 seedlings, g.	Concentration of ascorbic acid, mg./g. seedling
0·1 g. fructose + 0·5 g. mannose	0·27	0·596
0·5 g. arabinose + 0·5 g. mannose	0·15	0·553

The presence of mannose seems to inhibit growth altogether.

Summary.

1. The rate of production of ascorbic acid in pea seedlings germinated naturally has been estimated. The amount formed was found to rise for 2 days and then reach a constant concentration of 0·5 mg. of the acid per g. of the wet weight of the seedlings.

2. Embryo seedlings from peas were excised from the cotyledons and grown on nutrient gelatin. Seedlings cultivated on such a medium, containing hexoses, were found to have synthesised a large amount of ascorbic acid. Of the four hexoses examined, mannose gave the highest figure, 0·5 mg. of ascorbic acid per g. of seedling. Disaccharides also gave moderately high figures but all other substances examined either gave very low or absolutely negative values. It is suggested that hexoses may serve as precursors of vitamin C in germinating seeds.

3. The rate of production of ascorbic acid in the cultivated excised seedlings bore no direct relation to the rate of growth, *e.g.* seedlings grown on mannose showed very little growth but had a high concentration of ascorbic acid while arabinose and xylose seemed to favour growth but production of ascorbic acid was nil.

4. The concentration of ascorbic acid present per g. in the excised seedlings fell steadily during prolonged periods of cultivation.

5. The concentration of ascorbic acid formed was approximately proportional to the concentration of the substrate up to a certain optimum value, after which it remained constant.

6. Unsuccessful attempts were made to produce good growth combined with large production of ascorbic acid by cultivating excised seedlings on a medium containing a mixture of two substances—one of them found to favour growth and the other production of ascorbic acid.

I am very grateful to Dr L. J. Harris for his constant advice and valuable criticism. For his kind interest in this work, I wish to thank Sir F. G. Hopkins.

REFERENCES.

Birch, Harris and Ray (1933). *Biochem. J.* **27**, 590.
Brown and Morris (1890). *J. Chem. Soc.* **57**, 458.
Harris (1933). *Nature*, **132**, 27.
—— and Ray (1932). *Biochem. J.* **26**, 2067.
—— —— (1933). *Biochem. J.* **27**, 587.
Johnson (1933). *Biochem. J.* **27**, 1942.
Van Eekelen, Emmerie and Wolff (1934). *Acta Brev. Néerl.* 4, 3.

CXL. CHANGES IN THE CALCIUM AND MAGNESIUM OF THE SERUM AND IN THE INORGANIC PHOSPHORUS OF THE BLOOD OF COWS AT CALVING AND OF THE CALF DURING EARLY LIFE.

By WILLIAM MILLER ALLCROFT and WILLIAM GODDEN.

From the Rowett Research Institute, Aberdeen.

(*Received May 2nd, 1934.*)

In view of the recent work of Sjollema and his co-workers on "grass tetany," a good review of which is given by Sjollema himself [1932], and of the increasing incidence of "lactation tetany" or "Hereford disease" as referred to by Dryerre [1932], it was decided, in conjunction with Dr H. H. Green of the Ministry of Agriculture Veterinary Research Laboratory, Weybridge, to include the study of serum-Mg in normal cows throughout the reproductive cycle. We, therefore, repeated our studies [Godden and Allcroft, 1932] of the changes in the blood of the cow at calving, including determinations of serum-Mg in this series and omitting those of N.P.N., total chloride and sugar which had shown no marked alterations before.

The experimental procedure and the methods for serum-Ca and inorganic P were the same as used before except that, on this occasion, the calves were

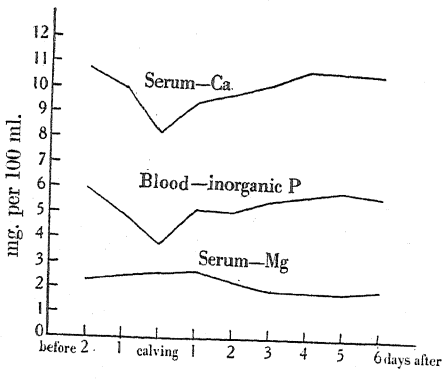

Fig. 1.

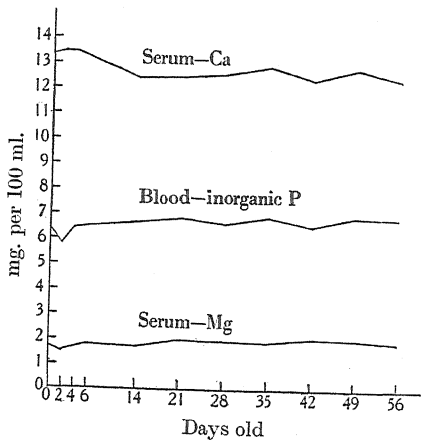

Fig. 2.

Fig. 1. Changes in the average composition of cow's blood just before, at and after calving (data for Cow 15 omitted).

Fig. 2. Changes in the average composition of the blood of 8 calves from birth to 8 weeks.

Table I. *Composition of the blood of cows, just before, at and for 6 days after calving.*

All values in mg. per 100 ml.

Days before or after calving	Serum-Ca	Serum-Mg	Blood-inorg. P	Serum-Ca	Serum Mg.	Blood-inorg. P	Serum-Ca	Serum-Mg	Blood-inorg. P	Serum-Ca	Serum-Mg	Blood-inorg. P
	Cow 4			Cow 5			Cow 7			Cow 8		
2 before	10·05	2·00	5·87	10·75	1·95	6·13	11·35	1·92	6·89	—	—	—
1 ,,	9·95	2·35	5·92	9·60	2·25	5·19	9·45	2·00	3·75	10·15	1·95	5·87
Calving	10·00	1·99	4·78	7·45	2·85	4·47	8·50	2·17	3·05	7·15	2·74	3·07
1 after	9·95	1·83	4·93	8·60	2·96	4·62	10·30	2·23	6·08	7·65	2·87	5·67
2 ,,	9·45	2·25	4·66	8·35	2·45	4·35	10·70	2·00	5·58	8·70	2·26	4·82
3 ,,	9·25	2·00	5·07	8·95	2·02	4·82	10·80	1·87	6·89	8·45	1·90	4·82
4 ,,	10·50	2·72	4·72	9·35	1·51	5·19	12·05	1·78	7·26	10·30	1·82	5·58
5 ,,	10·55	2·48	5·87	9·85	1·80	5·11	11·40	1·60	6·75	10·40	2·00	5·53
6 ,,	10·85	2·38	5·92	10·55	1·84	5·53	10·75	1·60	6·75	9·70	2·01	5·40
	Cow 11			Cow 12			Cow 13			Cow 15		
2 before	11·05	2·17	6·08	9·65	2·61	9·00	10·25	—	4·85	—	—	—
1 ,,	8·85	2·48	4·44	10·75	3·22	8·04	10·35	1·60	4·50	9·60	2·26	6·13
Calving	6·10	2·81	2·66	7·95	—	4·82	8·25	1·85	2·81	7·80	2·58	6·13
1 after	6·25	2·96	3·24	9·25	3·69	5·40	9·50	2·45	5·19	6·35	3·57	4·93
2 ,,	7·85	2·78	4·47	9·35	2·95	5·87	9·30	2·20	5·15	—	—	6·08
3 ,,	11·30	1·35	6·03	9·70	2·14	5·58	10·15	2·19	5·58	5·95	2·96	3·82
4 ,,	11·50	1·82	7·10	10·80	1·97	5·19	10·60	1·86	6·08	6·75	2·74	3·52
5 ,,	11·25	1·87	8·44	10·15	1·47	5·67	9·40	1·80	6·49	6·50	2·37	2·70
6 ,,	11·05	2·12	6·82	10·20	2·25	6·19	9·50	1·99	5·04	9·45	0·94	3·92
	Cow 16			Cow 18			Cow 19			Cow 20		
2 before	10·55	2·25	5·67	11·10	2·78	5·58	10·65	2·74	4·27	12·15	2·12	5·11
1 ,,	9·80	2·45	4·51	7·20	3·81	3·75	12·40	2·48	4·25	11·55	2·17	4·32
Calving	9·30	2·00	5·82	7·35	4·05	3·84	9·65	2·85	3·09	8·75	2·74	2·78
1 after	10·25	2·00	5·23	10·05	3·63	5·62	10·90	2·15	5·15	11·05	2·61	5·77
2 ,,	10·05	2·26	5·02	11·40	2·02	5·67	10·75	2·23	4·21	11·10	1·82	5·82
3 ,,	10·05	2·17	4·59	11·80	1·81	6·85	9·80	2·19	4·40	10·95	1·67	6·03
4 ,,	10·95	2·00	4·56	11·35	1·81	6·79	10·50	1·82	6·03	10·00	1·97	4·38
5 ,,	10·65	1·94	4·82	13·50	1·89	5·84	9·70	1·99	6·10	10·45	1·77	4·38
6 ,,	11·15	2·01	5·11	11·85	2·00	5·40	10·05	2·23	6·03	11·20	1·72	5·11

Table II. *Composition of the blood of 8 calves from birth to 8 weeks.*

All values in mg. per 100 ml.

Calf No.		Birth	2 days	4 days	6 days	14 days	21 days	28 days	35 days	42 days	49 days	56 days
4	Serum-Ca	13·85	14·25	13·20	12·90	12·35	12·35	12·75	11·95	11·35	11·40	11·90
	Serum-Mg	2·10	1·58	1·70	1·82	1·83	2·20	1·99	1·88	1·93	1·58	1·60
	Blood-inorganic P	6·43	6·08	5·87	4·78	6·37	5·72	6·61	6·65	6·23	6·46	6·31
5	Serum-Ca	13·55	13·45	13·45	14·40	12·95	12·95	12·80	13·60	13·10	13·10	12·95
	Serum-Mg	1·95	1·43	1·43	1·78	1·42	1·74	1·97	1·97	2·21	1·80	1·81
	Blood-inorganic P	7·34	6·08	6·82	7·58	8·34	7·10	7·42	7·58	6·40	8·14	7·34
7	Serum-Ca	14·00	14·15	13·85	12·80	12·35	12·40	13·35	12·65	12·30	13·95	12·60
	Serum-Mg	1·74	1·12	1·69	1·55	1·83	2·00	2·12	2·05	2·20	2·25	2·12
	Blood-inorganic P	6·55	6·19	6·08	7·10	7·18	7·94	7·84	8·44	7·42	6·43	6·68
8	Serum-Ca	12·85	13·30	—	12·75	11·75	13·45	12·35	12·55	12·75	12·05	11·55
	Serum-Mg	1·63	1·58	1·78	1·74	1·80	1·80	1·81	1·89	1·79	2·05	1·92
	Blood-inorganic P	4·35	5·19	6·55	6·13	6·25	6·82	6·75	5·97	6·37	6·55	6·70
11	Serum-Ca	13·20	13·15	12·45	12·25	13·00	13·30	12·55	14·05	12·80	13·50	12·30
	Serum-Mg	1·82	1·81	1·51	1·66	2·00	2·00	1·94	2·32	2·24	2·44	2·16
	Blood-inorganic P	8·24	6·08	5·62	6·03	5·62	7·68	6·82	7·18	7·26	6·89	7·46
12	Serum-Ca	12·70	14·15	14·45	14·20	12·45	11·75	12·05	13·10	12·55	12·05	13·75
	Serum-Mg	—	1·58	1·81	2·04	1·86	2·12	2·00	2·12	2·25	2·50	2·37
	Blood-inorganic P	4·47	6·19	6·37	6·13	6·37	6·37	5·87	6·43	8·24	7·58	7·54
13	Serum-Ca	12·95	12·05	13·80	12·60	12·00	11·25	11·95	12·15	11·90	13·90	12·25
	Serum-Mg	1·21	1·50	1·37	1·86	1·39	2·25	1·86	1·47	1·70	1·86	1·64
	Blood-inorganic P	6·37	5·15	7·50	6·82	6·75	7·10	6·03	6·75	6·25	6·75	6·55
15	Serum-Ca	13·70	13·05	13·20	13·75	12·50	12·25	12·75	13·05	12·30	12·65	11·90
	Serum-Mg	1·72	1·40	1·71	1·55	1·49	1·72	1·84	1·47	1·90	2·00	1·69
	Blood-inorganic P	7·50	5·62	6·55	7·50	6·82	6·08	5·67	6·13	4·47	6·68	6·43

studied for a longer period of time, namely 8 weeks. The method used for serum-Mg was a modification of that of Denis [1922] in which the phosphorus in the magnesium ammonium phosphate precipitate obtained from the Ca-free serum was estimated by the colorimetric method of Fiske and Subbarow [1925].

The data for each cow are given in Table I and curves of average values (excluding Cow No. 15) are given in Fig. 1. The data for 8 calves are given in Table II and the curves of average values in Fig. 2.

Discussion of results.

It should be noted that the basal ration of the whole of the cows was considered to be low in Ca. Of the cows, Nos. 5, 8, 12, 13, 15 and 18 received only this ration, whilst Nos. 4, 7, 11, 16, 19 and 20 received, in addition, a supplement of Ca salts to balance the ration. Whilst there are fairly wide variations between individual cows, these are not due to the dietary difference between the two groups, as may be seen by a study of the data in Table I.

As before, the tendency is for a fall in serum-Ca and blood-inorganic P just prior to parturition, with the lowest points at the time of calving: the only exception to this was Cow No. 16 in which the inorganic P was higher at calving. The labour of this cow was difficult and delayed and it is possible that true labour commenced 24 hours earlier, at the time of the lowest value for inorganic P. Thus we have confirmed our previous findings for the changes in serum-Ca and blood-inorganic P.

As mentioned above the data from Cow No. 15 were excluded from the averages. This was due to the fact that she suffered from a mild attack of milk fever which is reflected in the time taken for the serum-Ca to rise to its normal level and a corresponding high level of serum-Mg during that period.

Considering the results as a whole the behaviour of serum-Mg is more erratic than is that of serum-Ca or blood-inorganic P and the results are consequently less easy to assess. The curve in Fig. 1 reveals a tendency for the level of serum-Mg to be raised just at or for 24 to 48 hours after calving, but in some individual cows this rise occurred the day before calving. The post parturient fluctuations are similarly relatively wide. On the whole there appears to be a tendency for serum-Ca and serum-Mg to vary inversely.

In the case of the calves the data again showed a higher initial level of serum-Ca and blood-inorganic P than those normal for the dam. The serum-Ca dropped after 14 days from a level of 13·5–13·8 down to a level of about 12·4 and thereafter remained steady between 12·3 and 12·8, this being true for every calf except No. 11. Serum-Mg at birth was definitely of a lower order than that of the dam's normal and remained so for the first 14 days. After 21 days it had attained and remained fairly constant around the normal level for the cow.

Summary.

Earlier data on the decrease in serum-Ca and blood-inorganic P of cows just before and at calving are confirmed and data presented to show that, while the serum-Mg fluctuates rather widely about this period, it tends to rise to a higher level just before, at, or within 24 hours of calving. In general serum-Mg varies inversely with serum-Ca. Calves at birth and for the first 8 weeks of life show rather higher levels of serum-Ca and blood-inorganic P than the normals for

the dam, while the serum-Mg level at birth is lower than the dam's normal, but has attained this normal after 3 weeks.

We wish to acknowledge our indebtedness to Mr W. Thomson for assistance with the animals.

REFERENCES.

Denis (1922). *J. Biol. Chem.* **52**, 411.
Dryerre (1932). *Vet. Rec.* **12**, 1163.
Fiske and Subbarow (1925). *J. Biol. Chem.* **66**, 375.
Godden and Allcroft (1932). *Biochem. J.* **26**, 1640.
Sjollema (1932). *Nutr. Abstr. Rev.* **1**, 621.

CXLI. THE SALTING-OUT OF AMINO-ACIDS FROM PROTEIN HYDROLYSATES.

I. THE ISOLATION OF TYROSINE, LEUCINE AND METHIONINE.

By EVELYN MARY HILL and WILLIAM ROBSON.

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(*Received May 4th, 1934.*)

Requiring a supply of *l*-methionine for experiments designed for an attempt to throw light on the intermediate metabolism of this amino-acid, we were beset at the outset of our work with the problem of obtaining it in quantity. The useful and interesting paper by Pirie [1932] on its isolation from caseinogen hydrolysates had not then been published, and the only method which was likely to give us the amount deemed necessary for our purpose was that of synthesis. A quantity of the racemic acid was actually synthesised on the lines described by Barger and Weichselbaum [1931] with satisfactory results. It was soon realised, however, that resolution would involve so much material and time that we were obliged to turn to the reconsideration of Mueller's [1923] original method of isolating the active amino-acid and attempt to improve his yields.

At this stage of our investigation Pirie's paper appeared and we were able by his method to isolate a quantity of the active amino-acid. The serious difficulty in the procedure, as the author himself indicates, lies in the butyl alcohol extraction of the acid hydrolysate, both layers being so darkly coloured that at times it is impossible to distinguish the interface. It appears to us, moreover, that the method adopted for removing mercury from the mercury-methionine precipitate by heating the latter with a solution of barium hydroxide is liable to lead to loss of amino-acid. Mercuric oxide in hot alkaline solution is an excellent reagent for the rapid oxidation of amino-acids; we have avoided its use by decomposing the precipitate with hydrogen sulphide. It must be added, however, that Pirie [1933] has since described a second method which avoids the extraction by butyl alcohol. Of this we have had no experience.

In repeating Pirie's [1932] procedure a second time, we sought some means of purifying the acid hydrolysate before the butyl alcohol extraction. Pirie himself used lead acetate with only partial success. After several unsuccessful attempts with various reagents, we finally obtained a transparent and only slightly coloured solution by boiling the hydrolysate with a good grade of charcoal, the p_H of the solution having previously been raised to 4·5 by the addition of sodium hydroxide. On the occasion when this was done, the solution had to remain overnight before the extraction by butyl alcohol could be carried out. Next morning there had separated a copious crystalline precipitate which, as might be expected, consisted mainly of tyrosine. Leucine was also present, but to our great interest, the mixture, after alkaline fusion, gave an intense nitroprusside reaction. That this reaction was not due to cysteine was proved by boiling a portion of the solid with a solution of sodium plumbate, when no precipitate of lead sulphide was obtained. The probability that cystine is not present in the precipitate receives support from other directions. Pfeiffer and

Angern [1924], for example, showed that whilst ammonium sulphate can precipitate cystine from aqueous solution, sodium chloride will not do so; again, Okabe [1928] has found that the solubility of cystine in sodium chloride solution is considerably greater than in distilled water. Ruling cystine out in this way, it was apparent that the nitroprusside reaction was due to methionine, and it became clear that, if the above tyrosine complex contained methionine in any quantity, the problem of the isolation of the latter amino-acid would be considerably simplified.

The investigation was proceeding on these lines when our attention was drawn to a paper by Barnett [1933] on the salting-out of leucine from protein hydrolysates. It will be seen from Barnett's curve (Fig. 1) that the minimum solubility of leucine in saturated sodium chloride solution lies at p_H 2·4, at which point, as the author shows, the amino-acid is precipitated as dileucine hydrochloride. In studying Barnett's paper it appeared to us that methionine might reasonably be expected to be precipitated with leucine from saturated salt solution at p_H 2·4. Subjecting a caseinogen hydrolysate to Barnett's procedure, we found that methionine was actually present in the precipitate obtained at p_H 2·4, and that Barnett had apparently overlooked its presence therein. Unfortunately from our point of view, a sulphur determination carried out on the precipitate showed us that methionine was present in only very small amount.

Finding that Barnett's leucine fraction would not yield us methionine in promising yield, we turned to another aspect of this salting-out phenomenon. We have included in Fig. 1 a curve showing the results obtained by Sano [1926] in an investigation of the solubility of leucine in aqueous solution at different values of p_H. Comparison of the curves of Barnett and Sano shows that the effect of sodium chloride in reducing the solubility of leucine is at a maximum at p_H 2·4 and almost as great in the neighbourhood of p_H 6, *i.e.* at the isoelectric point of the amino-acid. From a knowledge of their chemical constitutions it can safely be assumed that the isoelectric points of leucine and methionine cannot be far removed from one another. The question remained whether the yield of methionine would be increased if the protein hydrolysate, after adjustment to p_H 6, were saturated with sodium chloride. An experiment based on this reasoning appeared worth undertaking.

The tyrosine-leucine-methionine mixture which had been precipitated after decolorising the protein hydrolysate at p_H 4·5 was dissolved in the minimum quantity of water and kept overnight. Next morning the tyrosine had separated in exceptionally good quality and yield. After filtration, the mother-liquor containing some leucine and methionine was added to the original bulk of the hydrolysate. The combined solutions, the p_H of which had been adjusted to 6 by the addition of sodium hydroxide, were then concentrated *in vacuo*. Ultimately crystals of sodium chloride began to separate; the evaporation was then stopped and the mixture placed in the ice-chest overnight. During this time the separated salt had settled to the bottom, whilst floating near the surface was a crystalline mass consisting, as shown by examination, chiefly of leucine and methionine. By decanting the solution from the salt, the separated amino-acids were collected, redissolved in water, and the methionine isolated according to Pirie's procedure. After a second precipitation, the yield of methionine, which, from sulphur determinations, was found to be of a high degree of purity, only amounted to some 0·6 % of the caseinogen used. Examination of the leucine-methionine solution from which the methionine had been precipitated showed that there still remained dissolved some of this amino-acid which had escaped precipitation by mercuric acetate. It was clearly evident that the conditions

for precipitating most of the methionine from the solution were not ideal for the purpose.

A series of semi-quantitative experiments on the following lines proved that this was not only true, but that it would probably be a matter of some difficulty, owing to the presence of other amino-acids, to regulate the conditions so that

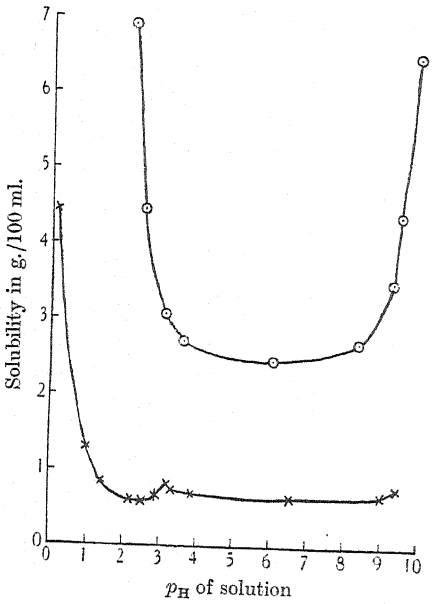

Fig. 1.

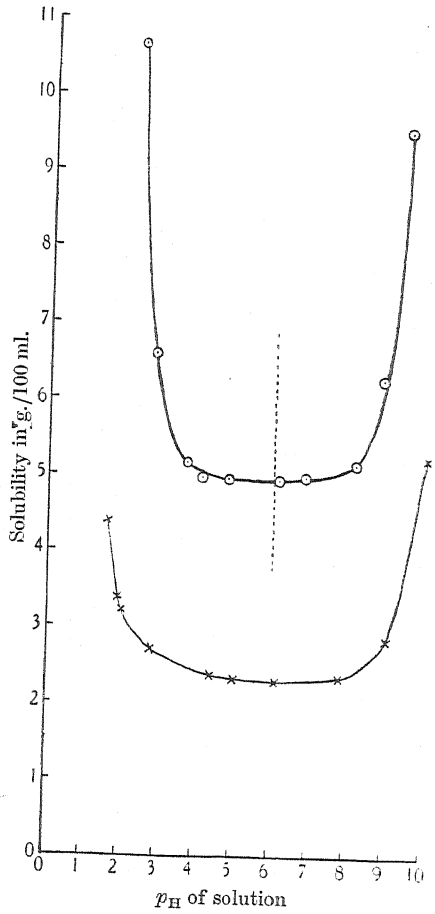

Fig. 2.

Fig. 1. ⊙—⊙ Solubility of leucine in water (Sano); ×—× Solubility of leucine in saturated sodium chloride solution (Barnett).

Fig. 2. ⊙—⊙ Solubility of methionine in water; ×—× Solubility of methionine in saturated NaCl solution.

most of the methionine would be precipitated. If, for example, to a solution of leucine, a solution of mercuric acetate in acetic acid be added drop by drop, a definite precipitation occurs. This precipitate however dissolves on the addition of (1) further mercuric acetate solution, or of (2) sodium chloride solution. Similar experiments with a solution of methionine showed that the precipitate produced by the addition of the mercuric acetate solution was soluble only in a

very much greater excess of the latter reagent, and that, while the presence of sodium chloride certainly favours the complete precipitation of the methionine, if the concentration of the sodium chloride exceeds a certain value, re-dissolution of the mercury-methionine precipitate occurs. Experimentation on these lines is being continued and will be reported in an early communication. At the present time it will suffice to add that if, after the precipitate obtained by treating the above leucine-methionine solution with mercuric acetate reagent has settled, sodium chloride solution be carefully added, a further precipitation takes place with the result that the ultimate yield of methionine obtained is raised from the above figure of 0·6 % to one of 1·5–1·6 %. Such yields have been obtained from caseinogen on several occasions. Egg-albumin, subjected to the same procedure, has given yields averaging 2·5 %.

The leucine was finally obtained by treating the solution, from which the mercury-methionine compound had been filtered, with hydrogen sulphide, filtering off the precipitated mercuric sulphide, concentrating the filtrate *in vacuo* to a syrup, dissolving the latter in the minimum quantity of water and raising the p_H to 6, when the leucine crystallises as the solution cools. Recrystallisation of the amino-acid follows in the usual way.

It was considered of general interest to include in this communication curves showing the solubility of methionine in aqueous and saturated sodium chloride solutions and these are shown in Fig. 2. The upper curve (solubility of methionine in aqueous solutions) appears to be symmetrical about an axis which cuts the p_H abscissa in the close neighbourhood of 6, a figure which may be taken as a close approximation to the isoelectric point of methionine. It is noteworthy moreover that the curve showing the solubility of methionine in saturated sodium chloride solution does not exhibit that dissymmetry which is seen in the corresponding curve for leucine (Fig. 1).

The average value of several actual yields of recrystallised amino-acids obtained from caseinogen (ash content 1·1 %, moisture 9 %, N 15 %) and egg-albumin (ash content 4·9 %, moisture 7·4 %, N 15·2 %) are as follows:

	Caseinogen %	Egg-albumin %
Tyrosine	3·0	1·35
Leucine	4·7	6·1
Methionine	1·55	2·5

Experimental.

1 kg. of caseinogen was hydrolysed with 2 litres of 20 % hydrochloric acid for 18–20 hours. The excess hydrochloric acid was removed in the usual way by concentration *in vacuo*, the residue being redissolved in hot water and evaporated until viscous.

Separation of tyrosine. The final residue was dissolved in 4 litres of boiling water, the solution treated with sufficient 40 % sodium hydroxide solution (approx. 460 ml.) to bring the p_H to 2·4, 40 g. of charcoal added, and the mixture, after being boiled for 20–30 minutes, was filtered hot. The charcoal-humin residue was extracted with about 1 litre of boiling water, and the filtered extract added to the main solution. The combined solution, which was quite clear and only faintly coloured, was allowed to cool overnight when the tyrosine crystallised out. This was filtered off and the filtrate used for the isolation of the methionine and leucine as described below. To increase the purity of the tyrosine thus obtained, it was recrystallised from boiling water in the usual way.

Separation of methionine. To the mother-liquor from the tyrosine, sodium hydroxide solution was cautiously added to p_H 6·0, and the resulting solution was evaporated under reduced pressure until sodium chloride commenced to separate (at approximately 1800 ml.). After cooling, the sodium chloride had settled to the bottom and the separated amino-acids were mainly floating near the surface of the liquid. The mother-liquor and the amino-acids were decanted to a Büchner funnel, and the acids sucked as dry as possible. The sodium chloride remaining in the container was separately dissolved in the minimum amount of water, and the solution thus obtained was used to wash the amino-acids on the Büchner free from mother-liquor.

The crude mixture of solid leucine and methionine obtained in this way was dissolved in 2 litres of water, and a cold saturated solution of mercuric acetate in 3 % acetic acid was added until no further precipitation occurred (about 250 g. mercuric acetate). After keeping for some time, a saturated solution containing approximately 60 g. of sodium chloride was added, when a further heavy precipitate formed. The mixture was kept overnight, and the mercury precipitate was then filtered off, suspended in about 2 litres of warm water and decomposed with hydrogen sulphide. The mercuric sulphide was boiled out with water, the mixture filtered and the filtrate added to the main methionine solution.

The solution containing the crude methionine was now evaporated almost to dryness, the residue dissolved in some 200 ml. of alcohol, the p_H of the alcoholic solution adjusted to 6·0 by the addition of pyridine and the mixture left in the ice-chest overnight. Next morning, the precipitate of crude methionine was filtered off, washed with alcohol to remove any pyridine and finally dried with ether.

For purification the product was redissolved in boiling water (1 litre for every 10 g. of crude methionine), a boiling saturated solution of mercuric chloride (10 g. of mercuric chloride per g. of the amino-acid) added, and the mixture, after being boiled for some minutes, was set aside until cold. The mercury precipitate then formed a solid mass, from which the mother-liquor was easily decanted. The solid was transferred to a mortar, ground up with water, the mixture filtered and the recovered solid suspended in warm water and decomposed with hydrogen sulphide. The further stages of the separation of the methionine followed the lines described above.

A further recrystallisation may be carried out from 75 % alcohol; it is inadvisable to use water since the solubility of methionine in this medium is approximately 5 % (Fig. 2). If, as sometimes happens, it is found difficult to remove the last traces of pyridine from the methionine, recrystallisation from 75 % alcohol, to which a few drops of acetic acid have been added, will enable this to be done.

Separation of leucine. The solution, from which the methionine was first precipitated by mercuric acetate, was freed from mercury by treatment with hydrogen sulphide. The mercuric sulphide was filtered off, the sulphide washed with boiling water, and the combined filtrates were concentrated *in vacuo* to a small bulk. The concentrate was dissolved in a small quantity of water and the p_H adjusted to 6·0, when the leucine at once began to crystallise. After some hours, the separated leucine was filtered off and recrystallised from dilute alcohol. This completed the procedure.

Summary.

A simple procedure is described for the isolation from caseinogen hydrolysates of tyrosine, methionine and leucine. The yields, especially those of tyrosine and methionine, obtained compare very favourably with figures already in the literature for these amino-acids.

The work forming the basis of this paper was done during the tenure by one of us (E. M. H.) of a Berridge Studentship awarded by the Delegacy of the University of London King's College.

REFERENCES.

Barger and Weichselbaum (1931). *Biochem. J.* **25**, 997.
Barnett (1933). *J. Biol. Chem.* **100**, 543.
Mueller (1923). *J. Biol. Chem.* **56**, 157.
Okabe (1928). *J. Biochem.* **8**, 441.
Pfeiffer and Angern (1924). *Z. physiol. Chem.* **133**, 180.
Pirie (1932). *Biochem. J.* **26**, 1270.
—— (1933). *Biochem. J.* **27**, 202.
Sano (1926). *Biochem. Z.* **168**, 14.

CXLII. AN EFFECT OF ASCORBIC ACID ON FATTY ACID OXIDATIONS IN THE LIVER.

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In the course of experiments on the oxidation of fatty acids by the liver of guinea-pigs it was found that the yield of acetoacetic acid appeared to depend on the state of nutrition of the animal. Preliminary experiments indicated that the livers of guinea-pigs fed for a lengthy period on a scorbutic diet behaved differently from the normal, a lower yield of acetoacetic acid being produced, and for this reason work was commenced on the effects of ascorbic acid on fatty acid oxidations of liver. Although further experiment has not shown a very strict line of demarcation between the activities of the livers of scorbutic and normal guinea-pigs, it has become abundantly clear that ascorbic acid exercises very definite effects on the respiration of, and on the power to oxidise fatty acids by, the livers of both guinea-pigs and rats.

It appears that the most significant effect of the addition of ascorbic acid to liver slices is the maintenance of respiration at a relatively high level for a much longer period than occurs in liver slices to which ascorbic acid has not been added. This is particularly noticeable with the rat where the respiration of a liver slice falls off quickly after an initial period of a fairly constant rate of oxygen uptake.

It is much less noticeable in the guinea-pig where the Q_{O_2} tends to remain constant over a long period; hence results with guinea-pigs are irregular and apparently much more dependent on nutritional conditions than is the case with the rat. Increases of acetoacetic acid production ranging from 30 to 200 % due to the addition of ascorbic acid have been noted with the rat and from 0 to 60 % with the guinea-pig; these increases in the yield of acetoacetic acid are probably closely connected with the maintenance of the relatively high levels in the rate of oxygen uptake, the oxidation of fatty acids being greatly dependent on the general respiratory metabolism of the liver cell.

Technique.

The technique described by the writers [1933] in their work on the oxidation of fatty acids in the liver has been followed. Measurements of oxygen uptake and acetoacetic acid yield (at 37°) have been obtained with the Barcroft differential manometer. Acetoacetic acid has been estimated manometrically by the method described by the writers [1933]. A small change in the technique of this estimation has been adopted: 1 ml. $M/5$ acetate buffer p_H 3·8 has been replaced by 1 ml. $M/2$ acetic acid. The results are equally accurate and consistent.

The acetoacetic yield is expressed by the symbol $Q_{\text{acetoacetic acid}}$ or Q_{ac} which represents the μl. CO_2 equivalent to acetoacetic acid produced by 1 mg. dry weight liver tissue per hour.

The only animals used in this work have been guinea-pigs (from various sources) and rats (also from various sources). We have recently confined ourselves, in work with rats, to the Wistar strain and find that this procedure is more satisfactory for obtaining comparable results.

The substrates under investigation have been butyric, crotonic and *dl*-β-hydroxybutyric acids. These have been chosen because they are very good sources of acetoacetic acid and also because the mechanism of their oxidation to acetoacetic acid is at present under investigation in this laboratory. A few experiments with higher fatty acids such as *n*-hexanoic acid have also been carried out and found to give results essentially similar to those found with butyric and crotonic acids.

Buffer solution. It was shown by the writers [1933] that concentrations of phosphate buffer (p_H 7·4) of the order $M/15$ appreciably diminish both Q_{O_2} and Q_{ac} of guinea-pig liver, and this was traced to the removal of calcium ions from the medium bathing the liver slices. Low concentrations of phosphate buffer solution do not buffer well in experiments lasting 3 hours or more. The difficulty of adequate buffering has been overcome (or partially so) without affecting the concentration of calcium ions by the use of sodium glycerophosphate buffer. Sodium glycerophosphate (B.D.H.) solution was brought to p_H 7·4 by addition of hydrochloric acid, and this was diluted by Locke solution and the substrate under investigation to make its final concentration $M/15$. Experience has shown that with both guinea-pig and rat liver slices of the order of 20 mg. dry weight there is a drop in p_H, in an experiment of 3 hours' duration, of about 0·2.

The final medium was made up as follows:

1·0 ml. $M/5$ sodium glycerophosphate solution (p_H 7·4).
1·5 ml. Locke solution[1].
0·5 ml. 0·1 M substrate solution.

Such a glycerophosphate-Locke medium has been used in the experiments described below.

Liver slices were added to this medium, and then 0·5 ml. of a neutral solution of ascorbic acid was added. All experiments with liver slices were carried out in an atmosphere of oxygen.

The manometric apparatus was allowed to shake at the rate of 90 times a minute, and readings were taken every 15 minutes.

After 3 hours, the liver slices were carefully removed, washed in distilled water and dried at 105°. Usually very little disintegration of the liver slice occurred. 2·5 or 3 ml. of the medium were then transferred to another Barcroft apparatus for estimation of acetoacetic acid. Determinations of final p_H were always made, and the results of any experiment where there had been a considerable change in p_H were discarded.

Choice of liver slices. It is important for the investigation of fatty acid oxidation with liver slices to select slices of appropriate sizes and thicknesses. It has been shown by the writers [1933] that mincing liver results in a cessation of fatty acid oxidation and it would be expected that very thin slices of liver

[1] Occasionally we found it desirable to use only 1 ml. Locke solution. Care was always taken, however, to use the same quantity of Locke solution in all vessels in any one experiment. The Locke solution was composed of 0·003 M $CaCl_2$, 0·0065 M KCl and 0·1445 M NaCl.

would approximate to the condition of minced tissue. Experience shows that liver slices less than 0·2 mm. thick give irregular results; the Q_{O_2} usually falls off rapidly and there is a diminished Q_{ac}. There is also an upper limit to the thickness of the slices used, determined probably by the diffusion of oxygen. Slices thicker than 0·4 mm. give a diminished Q_{O_2} and Q_{ac}. A few descriptive results are shown in Table I.

Table I. *Effects of varying thicknesses of liver slices (guinea-pig) on* $Q_{acetoacetic\ acid}$ *in presence of crotonate, 0·017* M. *(Glycerophosphate-Locke medium.)*

Exp.	Thickness mm.	Q_{ac}
1	0·28	2·3
	0·43	2·4
	0·56	1·6
2	0·29	2·0
	0·27	2·0
	0·67	1·1

We have therefore tried in all experiments to cut slices varying between 0·2 and 0·4 mm. Moreover in any one experiment where comparisons are made on one tissue it has been our endeavour to use slices of approximately the same thickness. Rat liver is more difficult to cut than guinea-pig liver and it is usually not an easy matter to obtain a large slice of uniform thickness. Hence we have used two or three small slices of approximately uniform thickness (as determined superficially by opacity or colour) rather than one large slice. Slices are always trimmed—a slice with frayed edges tends to disintegrate in the manometer vessel and to give, therefore, too low a weight after removal at the termination of the experiment.

The thickness of the slices is determined by floating them in a Petri dish containing saline, on the bottom of which is a sheet of squared paper. The area of the slice is outlined on the paper as carefully as possible and the area calculated in mm.^{2} With trimmed, rectangular slices the area can be obtained in this way with considerable accuracy. The thickness of a slice can now be calculated from the volume ($=5\times$ dry wt. in mg.) and the area (mm.^{2}). With experience, slices of thicknesses varying from 0·2 to 0·4 mm. can be chosen with ease.

The livers are cut immediately after removal from an animal which has been killed by cutting the throat and then well bled.

Source of ascorbic acid. Two samples of ascorbic acid have been used in the work; one was kindly presented to us by Prof. A. Szent-Györgyi. The other was kindly given to us by the Hoffmann-La Roche Co. according to whom the analytical figures were: $[\alpha]_D = +24°$; M.P. 188–189°; equiv. wt. (iodine titration) $=88$. The two samples behaved in the same way.

Differences between guinea-pig liver and rat liver.

Representative results indicating the respiration of, and acetoacetic acid production by, guinea-pig and rat livers are shown in Table II. Detailed results concerning guinea-pig liver oxidations are given in our earlier paper [1933] and will not be further referred to here. The most striking difference between rat and guinea-pig livers is the high Q_{O_2} of the former compared with that of the latter. Rat liver usually gives a Q_{O_2} of 10–12; guinea-pig liver gives a Q_{O_2} of 4–6.

Another difference, which will be commented upon again later, is that whilst the oxygen uptake of guinea-pig liver (under the experimental conditions

Table II. *Respiration of, and acetoacetic acid production by, liver slices from guinea-pig and rat.*

Substrates present as Na salts.

Exp.	Substrate (0·017 *M*)	Q_{O_2}	Q_{ac}
1. Guinea-pig liver	Nil	4·0	0·2
,,	Crotonate	9·0	3·6
,,	Butyrate	10·5	4·4
,,	*dl*-β-Hydroxybutyrate	6·1	1·8
2. Rat liver	Nil	11·6	0·8
,,	Crotonate	12·8	4·9
3. Rat liver	Nil	10·7	0·9
,,	Butyrate	12·6	3·0
4. Rat liver	Nil	9·5	0·7
,,	*dl*-β-Hydroxybutyrate	11·9	6·4

already described) is usually constant for 2 or 3 hours, that of rat liver begins to fall off rapidly after a preliminary period of fairly constant oxygen uptake which varies from 45 to 90 mins. As a general rule the thinner the slice the quicker is the tendency for the oxygen uptake to fall from a linear rate.

When sodium butyrate or sodium crotonate at a concentration of 0·014 *M* is added to guinea-pig liver there is a decided increase in Q_{O_2}, the figures in Table II being quite typical. The rise in Q_{O_2} with rat liver due to butyrate or crotonate is always very much less and we have had cases when the Q_{O_2} of rat liver has not been changed by the addition of butyrate or crotonate, although it was clear from the acetoacetic acid production that oxidation of the fatty acid had been proceeding.

The acetoacetic acid production from guinea-pig liver in presence of butyrate or crotonate is variable and seems to be dependent on the size of the liver. As a general rule the larger the liver in a well fed animal the smaller is the yield of acetoacetic acid. This observation will be commented upon at greater length in another communication. The acetoacetic acid production from *dl*-β-hydroxybutyric acid (0·014 *M*) with guinea-pig liver is much less variable than that from butyric or crotonic acid. With this animal the Q_{ac} is usually of the order 1·8 whilst that from butyrate and crotonate may vary from 1·5 to 4·5. With rat liver the yield of acetoacetic acid from *dl*-β-hydroxybutyric acid is considerably higher than with guinea-pig and the Q_{ac} is of the order 4–6. The Q_{ac} with rat liver from butyrate and crotonate is lower and has about the same range of variation as with guinea-pig.

We have observed that the rate of oxygen uptake tends to fall off to a greater extent in the presence of crotonate than in the presence of butyrate, and this seems to apply to both guinea-pig and rat.

Oxidation of ascorbic acid in the liver.

Harrison [1933] in his experiments on the effect of ascorbic acid on the respiration of scorbutic and normal guinea-pig tissue "balances" the ascorbic acid in the manometric vessels by placing the acid in phosphate buffer without tissue in the left-hand vessel of the Barcroft apparatus. This we have not done in our experiments because there is no proof that the rate of oxidation of ascorbic acid in the presence of tissue is equal to or is of the same order as that in the absence of tissue. This constitutes only a minor criticism of Harrison's results, for he used smaller quantities of ascorbic acid than we have used.

The following experiments indicate, with intact liver slices, that after 3 hours at 37° ascorbic acid is by no means fully oxidised, although it is so in the absence of tissue.

Exp. 1. 0·5 ml. of a neutral solution of ascorbic acid containing approximately 3 mg. per ml. was added to 3 ml. glycerophosphate-Locke medium and allowed to autoxidise in the presence of oxygen under the same conditions as were used for work with liver slices. After 3 hours 1 ml. of the medium was added to 4 ml. 3 % trichloroacetic acid and titrated with $N/200$ iodine solution. 0·04 ml. was required. The ascorbic acid was therefore practically completely oxidised under the given experimental conditions.

Exp. 2. 0·5 ml. of a neutral solution of ascorbic acid containing approximately 3 mg. per ml. was added to a glycerophosphate-Locke medium containing rat liver slices and to a glycerophosphate-Locke-crotonate (0·014 M) medium containing rat liver slices. After 3 hours' shaking in oxygen at 37°, the slices were removed and 1 ml. of each of the media was added to 4 ml. 3 % trichloroacetic acid and the solution titrated with $N/200$ iodine solution. The same procedure was carried out in control experiments where no ascorbic acid had been added. The following iodine titres were obtained:

			$N/200$ iodine titre ml.
(*a*) Glycerophosphate-Locke medium	Liver slices	No ascorbic acid	0·16
(*b*) Glycerophosphate-Locke-crotonate medium	,,	,,	0·12
(*c*) Glycerophosphate-Locke medium	,,	Ascorbic acid present	0·48
(*d*) Glycerophosphate-Locke-crotonate medium	,,	,,	0·49

An iodine titration on the ascorbic acid itself showed that 1 ml. of the medium would have required 0·95 ml. $N/200$ iodine had there been no oxidation of ascorbic acid. Hence from (*a*) and (*c*) 32/95, *i.e.* 33 % of the original quantity of ascorbic acid still remains and from (*b*) and (*d*) 38 % remains. Another experiment carried out under similar conditions showed that 49 % of the original quantity of ascorbic acid still remained at the termination of the experiment.

These experiments prove either (1) that the oxidation of ascorbic acid is retarded by liver slices, or (2) that oxidised ascorbic acid is reduced by liver slices, an equilibrium being established.

The second conclusion is the more likely one in view of Szent-Györgyi's experiments on the reduction of oxidised hexuronic acid by animal tissues [1928].

The action of ascorbic acid on the respiration of, and acetoacetic acid production by, normal guinea-pig liver.

According to Harrison [1933] ascorbic acid has but little effect on the respiration of normal guinea-pig liver. The probability is that there is a small but variable increase if the criticism mentioned above is taken into consideration. Our own experiments bear out this conclusion. Table III shows the effects of the addition of ascorbic acid[1] on the Q_{O_2} and Q_{ac} of normal guinea-pig liver slices in the presence of sodium crotonate or butyrate. It will be observed that the Q_{O_2} is in all cases raised, but the amount of rise is only about that to be expected by the oxidation of part of the ascorbic acid added. The expected change in Q_{O_2} (taken over 3 hours) for liver slices of 20 mg. dry weight is a rise of about 1·6 with an initial concentration of ascorbic acid of 0·5 mg./ml. but it is difficult to be certain of this as estimations of the amounts of ascorbic acid remaining at the end of the experiments were not made. It is clear that no significant increase attributable to the action of the added ascorbic acid on the oxidative metabolism of liver was obtained. Exp. 258 showed definitely a higher value of Q_{O_2} in presence of ascorbic acid but it is to be noted that the Q_{O_2} of the liver in this experiment in

[1] The ascorbic acid was neutralised with NaOH just before being added to the Barcroft vessels.

Table III. *Effects of ascorbic acid on the respiration of, and acetoacetic acid production by, liver slices of normal guinea-pig in presence of butyrate and crotonate.*

Substrate concentration 0·014 *M*. Glycerophosphate-Locke medium. Initial $p_H = 7{\cdot}4$. Final p_H = usually 7·1–7·2. Each experiment refers to a single animal.

Exp.	Weight of liver slices taken (mg. dry wt.)	Substrate	mg. per ml. ascorbic acid added	Q_{O_2} (av. over 3 hours)	Q_{ac}
128	24·3	Crotonate	Nil	9·5	3·1
	21·2	,,	0·43	10·4	3·5
139	24·2	Butyrate	Nil	8·5	3·0
	27·0	,,	0·50	9·6	2·9
140	38·8	,,	Nil	10·6	3·5
	34·5	,,	0·50	12·1	4·3
141	22·5	Crotonate	Nil	9·0	3·2
	18·5	,,	0·50	9·6	3·4
256	17·4	,,	Nil	7·6	2·4
	17·6	,,	0·86	9·8	3·2
258	14·6	,,	Nil	7·0	2·3
	12·1	,,	0·86	10·6	3·6

the absence of ascorbic acid was lower than usual; this was due to the fact that the rate of oxygen uptake fell off more rapidly than commonly occurred. In the presence of ascorbic acid the rate of oxygen uptake was constant throughout most of the experiment (see Table VI).

It will be noted that in most cases the presence of ascorbic acid produced an increase in Q_{ac}. The increase is usually small, but occasionally, as in Exp. 258, of the order of 50 to 60 %.

The results given in Table III are typical of a large number of experiments with normal guinea-pigs and show that whilst the effect of added ascorbic acid on the whole is small, irregularities (*i.e.* significant increases in Q_{O_2} and Q_{ac}) may occur.

Ascorbic acid and scorbutic guinea-pig liver[1].

The livers of guinea-pigs fed on a scorbutic diet for 15 days or longer usually show rises of Q_{O_2} and Q_{ac} in the presence of ascorbic acid, which are significantly greater than the average rises found with normal guinea-pigs. The animals were killed when they showed definite signs of scurvy and after their weights had reached a maximum and fallen 10–15 % of the maximum value. There is less irregularity about the results with scorbutic guinea-pig livers and an increase

Table IV. *Effects of addition of ascorbic acid on respiration of, and acetoacetic acid production by, liver slices from guinea-pigs fed on a scorbutic diet for varying periods.*

Substrate, crotonate 0·014 *M*. Conditions as in Table III.

Exp.	Period of scorbutic diet days	Weight of liver slices taken mg. dry wt.	mg. per ml. ascorbic acid added	Q_{O_2} (av. over 3 hours)	Q_{ac}
260	12	21·5	Nil	9·4	2·7
		21·0	0·86	11·2	3·3
264	15	17·6	Nil	8·6	2·9
		16·6	0·86	12·3	4·1
131	17	23·8	Nil	8·5	2·9
		24·4	0·50	10·1	4·0

[1] We are much indebted to the authorities of the Veterinary Laboratory, Weybridge, for supplying us with guinea-pigs in various stages of scurvy.

in Q_{ac} has always been found. The results of Table IV are typical. The percentage increase in Q_{ac} due to the addition of ascorbic acid is found to be always greater than the percentage increase in Q_{O_2}. It is worthy of note, however, that increases of the same order have been found occasionally with normal and apparently healthy guinea-pigs.

Ascorbic acid and normal rat liver.

Ascorbic acid has very definite effects on the Q_{O_2} and Q_{ac} of rat liver in the presence of sodium butyrate or sodium crotonate. The results given in Table V

Table V. *Effects of addition of ascorbic acid on the respiration of, and the production of acetoacetic acid by, normal rat liver in the presence of butyrate or crotonate.*

Substrate concentration 0·014 *M*. Conditions as in Table III.

Exp.	Weight of liver slices taken mg. dry wt.	Thickness of slices mm.	Substrate	mg. per ml. ascorbic acid added	Q_{O_2} (av. over 3 hours)	Q_{ac}
134	21·7	—	Butyrate	Nil	13·2	3·6
	25·5	—	,,	0·50	15·6	3·9
	17·2	—	Crotonate	Nil	10·0	2·5
	25·5	—	,,	0·50	14·3	4·4
160	22·8	—	,,	Nil	11·1	3·6
	20·5	—	,,	0·5	13·9	4·5
	18·3	—	,,	1·0	15·3	4·9
	19·7	—	,,	2·0	17·7	5·6
263	13·6	0·26	,,	Nil	12·2	3·1
	14·5	0·26	,,	0·86	13·0	4·3
266	14·0	0·27	,,	Nil	12·0	3·6
	12·8	0·26	,,	0·86	16·6	6·6
267	22·3	0·33	,,	Nil	12·4	3·2
	20·4	0·37	,,	0·86	13·2	4·5
268	16·6	0·30	Butyrate	Nil	14·1	3·2
	17·6	0·27	,,	0·86	16·5	4·5
	In the following experiments only Wistar rats were used.					
274	20·9	0·38	Nil	Nil	11·6	0·8
	19·6	0·37	Crotonate	Nil	12·9	4·9
	24·2	0·43	Nil	0·86	13·0	1·6
	20·2	0·39	Crotonate	0·86	16·7	8·4
275	13·6	0·24	Nil	Nil	8·0	0·5
	13·3	0·23	Butyrate	Nil	10·0	1·6
	16·5	0·25	Nil	0·86	11·2	1·0
	17·5	0·26	Butyrate	0·86	13·7	2·5
276	23·8	0·37	Nil	Nil	10·7	0·9
	22·4	0·33	Butyrate	Nil	12·6	2·9
	20·0	0·32	Nil	0·86	12·7	1·4
	18·3	0·30	Butyrate	0·86	17·8	5·3
283	15·9	0·28	Crotonate	Nil	9·4	1·8
	16·2	0·30	,,	0·43	15·6	4·1
	14·5	0·28	,,	0·86	15·1	4·5
	15·8	0·30	,,	1·3	14·7	5·1
285	20·3	0·22	Nil	Nil	8·4	0·3
	29·2	0·28	Crotonate	Nil	8·1	1·5
	20·7	0·23	Nil	0·43	12·2	0·4
	25·2	0·29	Crotonate	0·43	13·9	3·8
287	23·7	0·33	,,	Nil	11·3	2·2
	18·6	0·28	,,	0·43	15·1	2·6
288	26·8	0·33	,,	Nil	7·0	0·9
	20·0	0·30	,,	0·43	11·9	1·4

are typical of those found in about 50 experiments. The increases in Q_{O_2} and Q_{ac} are not regular and vary from rat to rat, but increases in Q_{ac} of the order of 80 % or more have often been found (see Exps. 134, 160, 266, 274, 276, 283, 285). Large increases in Q_{O_2} have also been obtained but as stated earlier these are more difficult to interpret owing to the fact that analyses of the ascorbic acid contents of the vessels at the termination of the experiments were not carried out. Rat liver usually gives a significant Q_{ac} in the absence of added substrates, and this value is invariably increased by the addition of ascorbic acid (see Exps. 274, 275, 276).

The problem now remains as to how ascorbic acid brings about the increased oxidation of butyric and crotonic acids—a very definite phenomenon in the case of rat liver and of scorbutic guinea-pig liver but doubtful, and at all events irregular, with normal guinea-pig liver. Control experiments have shown that the autoxidation of ascorbic acid does not induce oxidation of butyrate or crotonate (*i.e.* in the absence of tissue) and the negative results found with some guinea-pig livers confirm this.

It seems at first sight unlikely that ascorbic acid is essential for the oxidation of fatty acids, for scorbutic guinea-pig liver gives a Q_{ac} not far removed from normal. On the other hand no tests for ascorbic acid were carried out on the livers of the scorbutic animals and it is impossible for us to say what were the actual concentrations of ascorbic acid in the livers in question.

An explanation of this phenomenon, more satisfactory than one involving the assumption that ascorbic acid specifically affects fatty acid oxidation, comes from a consideration of the effects of ascorbic acid on the rates of oxygen uptake.

The effect of ascorbic acid on the rates of oxygen uptake by liver slices.

The rate of oxygen uptake by guinea-pig liver either alone or in the presence of crotonate and butyrate (0·014 *M*) is usually constant, the Q_{O_2} at the end of a 3 hours' experiment not being appreciably different from that half way through the experiment.

The presence of ascorbic acid does not alter the rate apart from a rise in Q_{O_2} at the commencement of an experiment owing probably to autoxidation of a portion of the ascorbic acid itself.

Rat livers, however, when slices are used of the thickness already quoted almost invariably show a fairly rapid fall in Q_{O_2} after an initial fairly steady rate of oxygen uptake, and at the end of 3 hours the Q_{O_2} may be less than 50 % of that half way through the experiment. The presence of ascorbic acid always diminishes this rate of fall, so much so that the rate of oxygen uptake may be the same at the end of 3 hours as it was at the end of 1½ hours. In Table VI an analysis of most of the results already quoted in earlier tables is given.

Turning to the first part of the table dealing with guinea-pigs it will be seen that the percentage decrease in Q_{O_2} at the end of the experiment from that half way through is small and not appreciably affected by ascorbic acid. The percentage increase in Q_{ac} due to the ascorbic acid is also small, though appreciable in Exp. 256. In Exp. 258, there is a 17 % drop in Q_{O_2} which is removed by the presence of ascorbic acid; in this experiment the percentage increase in Q_{ac} is highest (50 %). There is little doubt from our experience that had these experiments been carried on for a longer period the percentage decrease in Q_{O_2} in the absence of ascorbic acid would have been greater and the percentage increase in Q_{ac} due to ascorbic acid considerably larger.

Table VI. *A comparison between the effects of ascorbic acid on acetoacetic acid production and the rate of fall of Q_{O_2} of liver slices.*

Exp.	Substrate	mg. per ml. ascorbic acid added	% increase in Q_{ac} due to added ascorbic acid	% decrease in Q_{O_2} at end of 3 hours from that at end of 1½ hours
		Guinea-pigs (normal).		
128	Crotonate	Nil	—	0
	,,	0·43	13	0
129	Butyrate	Nil	—	10
	,,	0·5	0	10
139	,,	Nil	—	0
	,,	0·5	0	10
140	,,	Nil	—	10
	,,	0·5	22	10
141	Crotonate	Nil	—	0
	,,	0·5	6	0
256	,,	Nil	—	23
	,,	0·86	33	20
258	,,	Nil	—	17
	,,	0·86	57	0
		Rats.		
263	,,	Nil	—	70
	,,	0·86	39	0
266	,,	Nil	—	40
	,,	0·86	83	8
267	,,	Nil	—	20
	,,	0·86	41	8
268	Butyrate	Nil	—	40
	,,	0·86	41	11
274	Crotonate	Nil	—	70
	,,	0·86	71	15
275	Butyrate	Nil	—	30
	,,	0·86	55	9
276	,,	Nil	—	37
	,,	0·86	81	9
283	Crotonate	Nil	—	66
	,,	0·43	126	10
	,,	0·86	150	11
	,,	1·3	188	9
285	,,	Nil	—	67
	,,	0·43	153	15

Turning to the second part of the table dealing with the rats we observe percentage decreases in Q_{O_2} varying from 20 to 70, these decreases being practically eliminated by the presence of ascorbic acid. It is in these experiments that the largest effects of ascorbic acid in increasing Q_{ac} are to be found.

Figs. 1–4 illustrate the action of ascorbic acid on the rate of change of Q_{O_2} of rat liver slices throughout an experiment. Fig. 1 shows the action of ascorbic acid on the change of Q_{O_2} with time of rat liver slices in a glycerophosphate-Locke medium containing no added substrate. Figs. 2 and 3 show the effects of differing quantities of ascorbic acid on the respiration of rat liver in presence of sodium crotonate. Fig. 4 refers to the effect of ascorbic acid on the change of Q_{O_2} of rat liver in presence of sodium butyrate. The following points are of note:

(*a*) There is an initial sharp fall in the rate of change of Q_{O_2} in the presence of ascorbic acid, the Q_{O_2} then arriving at a fairly constant value. The initial sharp fall is probably due to autoxidation of part of the ascorbic acid added.

(*b*) The constant Q_{O_2} found at the end of an experiment in which ascorbic acid has been added to the liver slices is somewhat less than, but usually not far removed from, the initial rates of oxygen uptake found with those liver slices to which ascorbic acid has not been added.

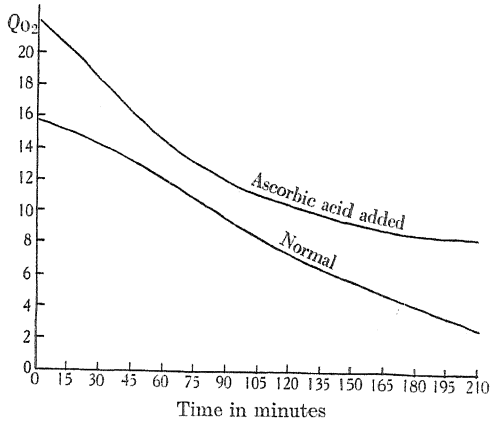

Fig. 1.

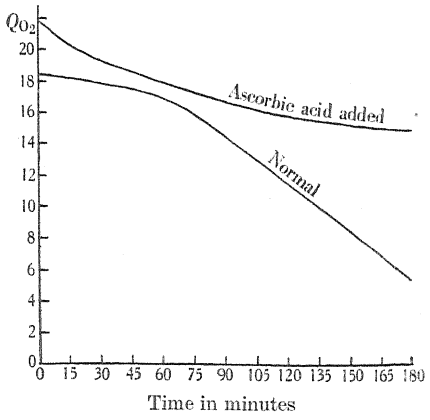

Fig. 2.

Fig. 1. Effect of addition of ascorbic acid (0·43 mg./ml.) on oxygen uptake by normal rat liver slices.

Fig. 2. Effect of addition of ascorbic acid (0·86 mg./ml.) on oxygen uptake of rat liver slices in presence of sodium crotonate (0·014 *M*).

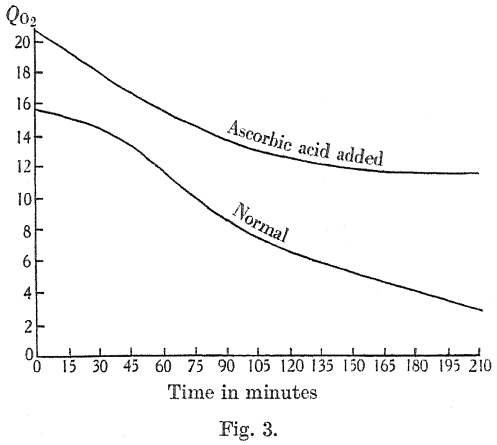

Fig. 3.

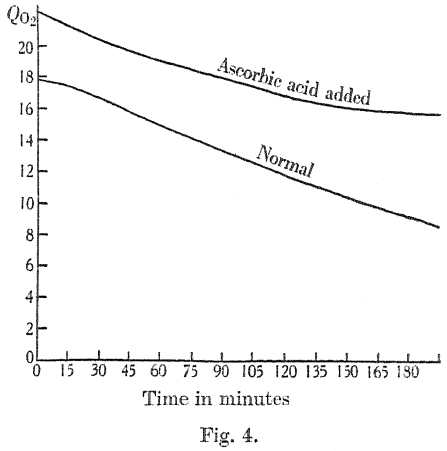

Fig. 4.

Fig. 3. Effect of addition of ascorbic acid (0·43 mg./ml.) on oxygen uptake of rat liver slices in presence of sodium crotonate (0·014 *M*).

Fig. 4. Effect of addition of ascorbic acid (0·86 mg./ml.) on oxygen uptake by rat liver slices in presence of sodium butyrate (0·014 *M*).

(*c*) Essentially the same phenomenon—a maintenance of a relatively high rate of respiration induced by the added ascorbic acid—occurs in the presence of either crotonate or butyrate and also in the absence of any added substrate.

The clearest inference from these results is that ascorbic acid plays a significant part in maintaining an intact respiratory metabolism in the liver. The efficiency of fatty acid oxidation depends upon the integrity of this respiratory metabolism. This hypothesis makes the action of ascorbic acid on fatty acid oxidation an indirect one but it serves to give a rational interpretation of the phenomena described. Further experiment alone can inform us whether ascorbic acid has a direct action on fatty acid oxidation or whether its action takes place *via* a number of intermediate steps. Such a hypothesis allows us to conclude that substances, other than ascorbic acid, and intimately associated with the respiratory metabolism of liver tissue may also affect fatty acid oxidation in a similar way; such a conclusion is obviously open to experimental verification.

Action of ascorbic acid on acetoacetic acid production by rat liver slices in presence of dl-*β-hydroxybutyric acid.*

In contrast to the effects of ascorbic acid on the oxidation of butyric and crotonic acids in presence of rat liver, there is little or no effect on the oxidation of *dl-β*-hydroxybutyric acid. Typical results are shown in Table VII. It follows

Table VII. *Effect of addition of ascorbic acid on the production of acetoacetic acid by rat liver slices in the presence of* dl-*β-hydroxybutyric acid.*

Exp.	Weight of liver taken mg. dry wt.	Thickness of slices mm.	Conc. of substrate *M*	mg. per ml. ascorbic acid added	Q_{O_2} (av. over 3 hours)	Q_{ac}
269	16·1	0·26	0·023	Nil	8·5	3·9
	16·3	0·28	0·023	0·86	10·8	4·3
270	14·4	0·27	0·023	Nil	13·4	6·3
	15·0	0·33	0·023	0·86	15·2	7·1
277	14·7	0·27	0·014	Nil	11·9	6·4
	15·8	0·29	0·014	0·86	13·8	5·8

that the oxidation of this hydroxy-acid does not depend upon the same mechanisms as those governing the oxidation of butyric and crotonic acids. This conclusion is supported by quite independent evidence, which it is hoped to report in due course in a further publication.

Action of iodoacetic acid on fatty acid oxidation by the liver.

It was found early in the course of our work on fatty acid oxidations by liver slices that iodoacetic acid (used as the sodium salt) exercised highly toxic effects at very low concentrations. Concentrations of the order of $N/5000$ will reduce the acetoacetic acid yield from butyrate or crotonate by over 50 %. The toxic action of the halogen-acid is a progressive one, the apparent inhibiting action on acetoacetic acid yield increasing with the length of exposure of the tissue to the iodoacetic acid.

On making a closer study of this phenomenon it was found that the apparent inhibitory effect of iodoacetic acid was greatly dependent on the thickness of slices used. The thicker the slice the smaller was the inhibition of acetoacetic acid production and experience showed that the most suitable thickness of slices to be used in demonstrating the highly toxic action of iodoacetic acid in an experiment of 3 hours' duration was of the order 0·2–0·3 mm. (using iodoacetic acid at a concentration about $N/5000$). Thinner slices than these suffered very rapid poisoning by the halogen acid. A series of typical results is shown in Table VIII.

Table VIII. *Action of iodoacetic acid on the production of acetoacetic acid by liver slices in presence of sodium crotonate (0·014 M).*

Exp.	Animal	Weight of liver slices taken mg. dry wt.	Thickness of slices mm.	Conc. of iodoacetic acid	Q_{O_2} (av. over 3 hours)	Q_{ac}
230	Guinea-pig	15·2	—	Nil	9·4	4·0
		15·9	—	N/6000	6·0	1·3
254	,,	22·7	0·33	Nil	7·7	1·8
		11·8	0·15	N/6000	3·4	0·3
		22·1	0·38	N/6000	7·8	1·4
		32·9	0·58	N/6000	7·7	1·8
255	,,	27·8	0·41	Nil	6·4	2·1
		31·9	0·38	N/7000	7·2	2·1
256	,,	17·4	0·22	Nil	7·6	2·4
		12·7	0·21	N/5250	5·9	1·1
257	Rat	8·6	0·18	Nil	13·9	4·3
		13·1	0·24	N/5250	5·7	1·6
258	Guinea-pig	14·6	0·26	Nil	7·0	2·2
		11·8	0·23	N/5250	3·0	1·3
260	,,	21·5	0·30	Nil	9·0	2·7
		21·3	0·27	N/5250	7·7	1·4

Attention may be drawn to Exps. 254 and 255 where it will be seen that with slices about 0·4 mm. thick but little change is shown in Q_{ac} in the presence of iodoacetic acid. Concentrations of iodoacetic acid higher than N/5000 will of course affect even the thicker slices. Exps. 256, 257, 258, 260 show that with slices of 0·2–0·3 mm. thickness, N/5250 iodoacetic acid brings about a drop in Q_{ac} of the order of 50 %.

Q_{O_2} is also decreased by the iodoacetic acid. A study of the rates of oxygen uptake of guinea-pig liver slices exposed to iodoacetic acid shows that the rate of oxygen uptake does not fall off immediately; the rate remains approximately constant for a variable period—45 to 90 mins.—and then falls rapidly. The phenomenon is reminiscent of that obtaining with rat liver slices (*i.e.* in the absence of iodoacetic acid). It could be explained by assuming that iodoacetic acid reacts slowly in the liver slice with some substance essential for the respiration of liver (and possibly for fatty acid oxidation) and that when the concentration of this substance falls below a certain limit there occurs a rapid fall in Q_{O_2} and Q_{ac}.

Action of ascorbic acid on guinea-pig liver poisoned by iodoacetic acid.

In view of the results already obtained with rat liver it was decided to investigate the action of ascorbic acid on normal guinea-pig liver slices exposed to iodoacetic acid.

It was found at once that the addition of ascorbic acid had precisely the same action on guinea-pig liver poisoned by iodoacetic acid as on normal rat liver. There was a prolongation of the steady rate of oxygen uptake and a decided increase in Q_{ac}. Representative results are shown in Table IX. In the presence of small concentrations of iodoacetic acid the decrease in Q_{O_2} at the end of 3 hours from that at the end of the first hour is 80 % or over. This decrease is lessened to the extent of about a half by the presence of ascorbic acid—a fact which is reflected in the increased average Q_{O_2}. The Q_{ac} may be increased as much as 200 % by the presence of ascorbic acid. We have never found however that ascorbic acid entirely neutralises the action of iodoacetic acid—the main effect, as already stated, appears to be a prolongation of the

Table IX. *Action of ascorbic acid on the production of acetoacetic acid by normal guinea-pig liver slices poisoned by iodoacetic acid.*

Substrate, sodium crotonate 0·014 *M*. The ascorbic acid was added at the same time as the iodoacetic acid to the liver slices (*i.e.* at the commencement of the experiment).

Exp.	Weight of liver slices mg. dry wt.	Thickness of slices mm.	Conc. of iodoacetic acid	mg. per ml. ascorbic acid added	Q_{ac}	Q_{O_2}	% decrease in Q_{O_2} at end of 3 hours from that at end of 1 hour
226	19·3	—	*N*/3000	Nil	0·9	4·9	80
	23·0	—	,,	0·43	2·5	8·2	40
251	13·3	0·21	*N*/7000	Nil	3·3	8·9	86
	16·7	0·22	,,	0·86	4·4	13·7	54
256	12·7	0·21	*N*/5250	Nil	1·1	6·5	80
	16·7	0·25	,,	0·86	2·2	8·5	42

maintenance of a relatively high rate of oxygen uptake. Experiments have failed so far to indicate a reaction between iodoacetic acid and ascorbic acid such as that which occurs between iodoacetic acid and glutathione.

These experiments with iodoacetic acid serve to confirm the conclusions already reached on the mechanism of action of ascorbic acid. If ascorbic acid were directly concerned with fatty acid oxidation and the toxic effect of iodoacetic acid were due to a reaction with ascorbic acid, then it would be possible to neutralise the action of the halogen acid by placing it in contact with the ascorbic acid. Such a neutralisation occurred with glutathione and iodoacetic acid in the case of oxidation of glucose by brain tissue [Quastel and Wheatley, 1932]. Ascorbic acid, however, does not neutralise the action of iodoacetic acid—even when present in considerable excess—it simply diminishes the rapidity of action of the latter.

One inference would be that iodoacetic acid in the liver slice removes a substance from the respiratory system, the production of which is greatly influenced by the presence of ascorbic acid. The substance in question would not only be essential for the maintenance of the normal respiratory level but also for the efficiency of fatty acid oxidation in the liver.

Action of glycogen on fatty acid oxidations.

In our previous paper [1933] we reported an inhibitory action of glycogen on acetoacetic acid production by guinea-pig liver slices. Over 12 experiments were carried out with consistent results. Repetition of this work 9 months later both with the same sample of glycogen and with a new sample of glycogen has failed to confirm this inhibition and at present we are at a loss to understand this irregularity of results. Our former conclusion, however, that added carbohydrate does not increase fatty acid oxidation in liver slices is not affected. Analysis of many of the livers used in the work described in this paper has not shown any distinct correlation between Q_{ac} and the glycogen content of the livers.

Summary.

1. Ascorbic acid, which is fully oxidised at 37° at p_H 7·4 in presence of oxygen, is only partially oxidised under the same conditions in presence of liver slices.

2. The presence of ascorbic acid secures an increase in Q_{O_2} and $Q_{\text{acetoacetic acid}}$ (in the latter case ranging from 30 to 200 %) of rat liver slices, when these are in a glycerophosphate-Locke medium containing sodium butyrate or crotonate.

3. The presence of ascorbic acid secures a significant increase in $Q_{\text{acetoacetic acid}}$ of scorbutic guinea-pig liver slices in media containing butyrate or crotonate; with normal guinea-pig liver slices, the increase in $Q_{\text{acetoacetic acid}}$ is small and irregular.

4. Ascorbic acid brings about a prolongation of the maintenance of the steady rate of oxygen uptake by guinea-pig and rat liver slices.

5. It is suggested that the effects of ascorbic acid on $Q_{\text{acetoacetic acid}}$ are due to the oxidation of fatty acids (*e.g.* butyric and also crotonic acids) being inherently connected with the general respiratory metabolism of the liver cell, and that ascorbic acid is important in maintaining this metabolism at a normal level.

6. Ascorbic acid appears not to affect the oxidation of *dl*-β-hydroxybutyric acid in presence of rat liver slices.

7. Iodoacetic acid at concentrations of the order of $N/5000$ has a large inhibitory action on the production of acetoacetic acid from butyric or crotonic acid by liver slices. This inhibition is reduced by the presence of added ascorbic acid, the reduction not being due to a combination between iodoacetic acid and ascorbic acid.

8. The action of glycogen on acetoacetic acid production by liver slices is discussed.

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REFERENCES.

Harrison (1933). *Biochem. J.* **27**, 1501.
Quastel and Wheatley (1932). *Biochem. J.* **26**, 2169.
—— —— (1933). *Biochem. J.* **27**, 1753.
Szent-Györgyi (1928). *Biochem. J.* **22**, 1387.

CXLIII. THE POLYURONIDE CONSTITUENTS OF FORAGE GRASSES.

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During the past few years, an extended study has been made in these laboratories of the chemistry of certain constituents of the forage grasses, the nitrogenous substances and the fats and waxes having received particular attention. Samples of a number of grasses of pure strain and known history being available, the opportunity was taken to examine the cell-wall substances of certain of these. Detailed information on the chemistry of these substances is extremely scanty, being limited to such rather empirical data as "crude fibre," "pentosan" and cellulose content of the tissues; in view of the importance of the cell-wall materials from the standpoint of nutritional value and digestibility of the crops, a much more detailed study seemed to be called for. The polyuronides (pectins and hemicelluloses) of cocksfoot grass have been examined in some detail, while a number of analyses of the chief cell-wall and other carbohydrate constituents of other grasses, grown under comparable conditions, are recorded.

Experimental.

Material used.

The cocksfoot used in the large-scale preparations of pectin and hemicellulose was obtained in the first place from the Agricultural Research Station of Imperial Chemical Industries Ltd., at Jealott's Hill. The crop was taken 3 weeks after the plot had been closely cut and dressed with nitrogenous fertiliser; the grass at this time was from 8 to 10 in. high, and the upper 6 in. of the blades were removed with a scythe. The first sample (1930) was minced, pressed and the residue extracted with ether and boiling alcohol, as described by Pollard *et al.* [1931]; this material was used only for preliminary experiments. The later batches (1931) were oven-dried, ground and extracted with ether only, so that the whole of the pectin, *etc.* remained in the tissue.

The material of the 1933 series was grown at the Imperial College Biological Field Station, Slough; it was allowed to grow to about 1 ft., cut low with a scythe and dried rapidly in a large steam-oven; the material, after grinding, was used without ether extraction. The small samples of various grasses used for the comparative analyses were also grown at Slough; their history is detailed below (p. 1034).

In all cases, in both large- and small-scale work, the ground dried grass was passed through sieves, and the portion of 60–90 mesh used for the extractions, experiments having shown that this was the most suitable size of particle.

Pectic substances.

250 g. of dried cocksfoot (1930) were extracted with 0·5 % ammonium oxalate on a boiling water-bath for 20 hours. The filtrate was precipitated with excess of alcohol, the gelatinous precipitate was redissolved in about 500 ml. of warm water and again precipitated with alcohol containing a little hydrochloric acid. After thorough washing with graded strengths of alcohol and drying, the precipitate was obtained as a nearly white powder (about 4 g.), containing 1·5 % of ash. Determination of the true pectin content of the substance by the calcium pectate method gave a calcium pectate value of 81 %. A sample of the calcium pectate prepared from the material gave furfuraldehyde 19·50 %, uronic anhydride ($CO_2 \times 4$) 67·10 % (average), agreeing with the values accepted for pure calcium pectate. The grass pectin was therefore considered to be of normal type.

Estimations of the amounts of the individual pectic substances present in certain of the samples were made, following the methods outlined by Branfoot [1929]. It was found necessary to vary the procedure of Branfoot in one respect. Her method includes extraction of the pectin present in the cell-wall as protopectin by means of boiling $N/75$ hydrochloric acid and estimation of the pectin in this solution as calcium pectate directly, *i.e.* without the preliminary precipitation with acidified alcohol. Unless this precipitation was carried out, the values found for "protopectin" were considerably higher than those for "total pectin" (ammonium oxalate extract), *i.e.* the $N/75$ hydrochloric acid removed from the tissue some substance capable of giving an insoluble calcium salt under the conditions of the usual calcium pectate precipitation (see below, p. 1030). It may be pointed out that this difficulty would not have arisen with the alternative methods of extraction proposed by Nanji and Norman [1928].

The results of some of these estimations are given in Table I.

Table I. *Distribution of pectic substances.*

Results expressed as g. of calcium pectate per 100 g. dry grass; mean of 3 or more values; see also Table V.

1	2	3	4	5	6
Sample no.	Total pectin (amm. oxalate extr.)	Pectin in HCl extr., (after alc. pptn.)	Pectin of middle lamella *etc.* (2–3)	"Pectin" in HCl extr., (omitting alc. pptn.)	Substance other than pectin in (5)
1930, Q	2·80	—	—	3·56	0·76
1931, 3	1·74	0·31	1·43	3·04	2·73
1931, 4	1·76	0·28	1·48	1·03	0·75

Methoxyl determinations were made on samples of pectin prepared from certain of the ammonium oxalate extracts by precipitation with acid alcohol and purified by reprecipitation, *etc.* The methoxyl values obtained were all below 3 %, after allowing for the non-pectic substances present as impurities in the material, on the basis of the calcium pectate values of the solids [*cf.* Norman, 1928]. It may be seen, however, from Table I, that by far the greater part of the pectin was extracted only by ammonium oxalate, *i.e.* occurred in the tissue as "middle lamella pectin" [Branfoot, 1929], which is usually regarded as being in the de-esterified or pectate form.

In order to obtain some further idea as to the degree of esterification of the pectin present otherwise than in the form of pectate, the following method was adopted. Ammonium oxalate extracts of the dried grass were made, the period

of extraction usually not exceeding 4 hours, at 98°; the extracts were precipitated with neutral alcohol, so that the insoluble pectic acid was not formed. The precipitate (containing ammonium pectate and soluble pectin) was well washed with 70 % alcohol and redissolved in water. In an aliquot portion of the solution, total pectin was determined in the usual manner. A further aliquot portion was treated directly with 0·4 *N* acetic acid and calcium chloride, without previous de-esterification with alkali; thus only the pectin present in the extract in the form of soluble pectate was precipitated as the calcium salt. In this manner it was possible to obtain an approximate estimate of the relative proportions of esterified and non-esterified pectin in the ammonium oxalate extracts; the assumption is made that no esterified pectin is precipitated by calcium chloride in the presence of acetic acid.

The results of these determinations, summarised in Table II, indicated that the major part of the pectic substances was indeed present in the de-esterified form. A recalculation of the methoxyl values of the esterified fractions of the various pectins, assuming that methoxyl groups were entirely absent from the "pectate-pectin" fractions, showed that (except in a single case) the average methoxyl content of the esterified pectin was still far below that required for the fully methylated derivative (11·76 % OMe). In three of the cases recorded, the calculated value was close to that required by the dimethoxyl ester, but this probably represented only a chance average figure. The unusually low methoxyl value found for sample 1930, Q, was probably due to the fact that in this case the ammonium oxalate extraction was allowed to continue for 20 hours; Norris and Schryver [1925] have shown that ammonium oxalate may produce considerable de-esterification of pectin during such prolonged periods of extraction.

Table II. *Methoxyl content of pectins.*

1	2	3	4	5
Sample	Ester-pectin as % of total pectin	Pectate-pectin as % of total pectin	Average OMe content of total pectin	OMe content of ester-pectin, %
1930, Q	40	60	0·6	1·5
1931, 3	34·5	65·5	1·93	5·59
1931, 4	21	79	1·18	5·62
1932	30	70	1·90	6·33
1933, G	24	76	2·80	11·50

It has been pointed out above (Table I) that precipitation of the extract given by *N*/75 HCl, without preliminary precipitation by acid alcohol, gave very high values for "calcium pectate," suggesting the presence of some substance giving an insoluble calcium salt under the conditions of the calcium pectate precipitation. A portion of the grass was allowed to stand at room temperature with *N*/75 HCl for 4 weeks, the acid being changed five times during the period; the acid extracts gave no trace of an insoluble calcium salt when tested. It seemed therefore that the original contaminant was not an acid liberated from an insoluble salt by the action of the *N*/75 HCl (*e.g.* oxalic acid from calcium oxalate), but that mild hydrolysis was definitely necessary for its production. Apart from this observation, the nature of the substance has not been investigated. The need, in dealing with leaf extracts at any rate, of performing a preliminary alcohol precipitation on the *N*/75 HCl extracts, as well as on those of ammonium oxalate, was evident from these results; it is not indicated by Branfoot [1929].

Hemicelluloses.

In comparison with the chemistry of the pectins, that of the hemicelluloses remains obscure; they comprise a group of substances without any very distinctive common property, and the most usual definition classes them simply as carbohydrate substances, not removed from plant tissues by water, but extracted by 4 or 5 % alkali. Many of the isolated hemicelluloses themselves are however water-soluble.

For the preparation of fairly large amounts of the hemicelluloses, the procedure of Norris and Preece [1930] was followed, and the fractions obtained have been designated according to their nomenclature. Batches of 250 or 500 g. of the dried cocksfoot were extracted first with cold water for 24 hours, then with 0·5 % ammonium oxalate for 6 hours at 98°. After washing with hot water, the well pressed residues were allowed to stand overnight with cold 50 % alcohol, containing 1 % NaOH, allowance being made for water retained in the residues [*cf.* Preece, 1931]. The alcoholic liquor was pressed out and the residue allowed to stand for several days with successive portions of 4 % sodium hydroxide solution at room temperature. The combined extracts were acidified with acetic acid, whereby the crude hemicellulose A was precipitated as a thick green flocculum, whose colloidal nature rendered its removal a matter of extreme difficulty, even in the presence of considerable excess of acetic acid. The most satisfactory separation was obtained by filtering several times on paper-pulp, on the surface of which was spread a thin layer of animal charcoal; a clear filtrate was thus obtained, while the hemicellulose A was recovered by extracting the pulp with alkali and reprecipitating with acetic acid. Considerable loss was however unavoidable at this stage. The hemicellulose was dried by means of alcohol, whereby much colouring matter was removed; the final product was still brown in colour and was found to contain 3 % of ash and 2·5 % N (Kjeldahl), equivalent to approximately 15 % of protein. Further purification was carried out by conversion into the copper compound, according to the method of Baker and Pope [1900], and products were obtained with 0·18 to 0·35 % ash and from 0·4 to 0·5 % N; further purification by this method seemed impossible.

After removal of hemicellulose A, the clear filtrates were treated with half their volume of acetone, to precipitate fraction B, and finally with excess of acetone, to precipitate fraction C; the crude fractions were in most cases converted directly into their copper compounds, and thus further subdivided into fractions B 1, B 2 and C 1, C 2 [Norris and Preece, 1930]. These substances, when dry, were obtained as cream-coloured or white powders, possessing the usual characteristics of their class; with the exception of B 1, they were readily soluble in cold water, giving light yellow solutions. B 1 was soluble only in dilute alkali but was not then reprecipitated on neutralisation. The fractions were free from N but still contained amounts of ash up to 1 %. The total yield of isolated hemicelluloses amounted only to about 60 % of the amount present in the grass, as estimated on a small sample (see Table V). From 1100 g. of dried grass the yields of the individual fractions were: A, 33 g.; B 1, 18·8 g.; B 2, 4·5 g.; B (unfractionated), 17 g.; C 1, 8·7 g.; C 2, 13·2 g.; C (unfractionated), 9·5 g.; total, 104·7 g. The yield was expected to amount to about 200 g.; the greatest losses occurred (*a*) in the isolation of fraction A and (*b*) during the purification by means of the copper compounds.

Estimations of furfuraldehyde yield and carbon dioxide yield (on distillation with 12·5 % HCl) were made by the ordinary methods on each fraction.

Measurements of rotation were also made, in the case of fractions A and B 1, in 2 % sodium hydroxide solution, in the other cases in aqueous solution. Only in the case of one sample of hemicellulose A was it possible to determine the rotation, on account of the opacity of the alkaline solutions. The results, given in Table III, are the mean of two or more determinations in each case and are calculated on an ash-free basis.

Table III. *Hemicelluloses of cocksfoot.*

1	2	3	4	5
Fraction	Grass sample	Furfuraldehyde yield %	Uronic anhydride % ($CO_2 \times 4$)	Rotation, $[\alpha]_D$
A	1931, 2	58·4	1·6	−102° (c = 1·5 %, in 2 % NaOH)
B 1	1931, 3	53·1	10·6	−47°
	1933, G	52·0	10·8	−51° (c = 1·0 %, in 2 % NaOH)
B 2	1931, 3	16·4	3·4	−53°
	1933, G	—	2·8	−12° (c = 1·4 %)
C 1	1931, 3	54·0	6·5	−83°
	1933, G	53·4	7·0	−79° (c = 1·4 %)
C 2	1931, 3	45·4	10·0	−66°
	1931, 4	—	—	−69°
	1933, G	43·6	9·8	−68° (c = 1·2 %)

In order to identify the sugar constituents of the hemicelluloses, portions of each were hydrolysed by boiling for 4 hours with 3·5 % sulphuric acid; excess of acid was removed by addition of the calculated amount of barium hydroxide solution. The filtrate in each case (distinctly acid except in the case of fraction A) was evaporated to small bulk, exactly neutralised with barium hydroxide and further evaporated to a syrup at low temperature; the syrup was extracted with warm 90 % alcohol. The alcoholic extract was evaporated again, the residue taken up in water, any barium present removed exactly and the liquid allowed to evaporate slowly *in vacuo*. In all cases except B 2, crystals readily formed in the syrups; these were removed and examined separately. The syrups were also examined for hexoses and pentoses by the usual methods. The qualitative examination of the five fractions for sugar constituents is summarised in Table IV.

Table IV. *Constituents of cocksfoot hemicelluloses.*

Fraction	Sugars, *etc.* present	Identified by	
A	Xylose	Rotation, $[\alpha]_D$ +19°. Cd xylonobromide. Osazone	Xylose crystals readily
	Arabinose	Diphenylhydrazone, M.P. 201°	
B 1	Xylose	As above	Crystals pure xylose
	Arabinose	,,	
	Galacturonic acid	Mucic acid, by HNO_3, M.P. 218°	
B 2	Galactose	Mucic acid, methylphenylhydrazone, M.P. 189°. Osazone (form of)	No crystals in syrup
	Arabinose	Diphenylhydrazone, M.P. 200°	
C 1	Xylose	As B 1, above	Xylose crystals readily
	Arabinose	,,	
	Uronic acid (?)		
C 2	Galactose	Methylphenylhydrazone, M.P. 189°. Mucic acid, M.P. 218°	Xylose crystals slowly from syrup
	Arabinose	Diphenylhydrazone, M.P. 201°	
	Xylose	Rotation, $[\alpha]_D$ = +19·5°. Cd xylonobromide	
	Galacturonic acid	Mucic acid, M.P. 218° by Br oxidation	

The identification of the uronic acid constituents of the fraction was a matter of more difficulty; in no case did the uronic anhydride content exceed 11 %, and, as is well known, the free uronic acids are very readily decomposed by dilute acid during hydrolysis. In the case of fractions A and B 2, no attempt was made to identify the very small amounts of uronic acid present. In the other cases, use was made of the bromine oxidation method of Heidelberger and Goebel [1927], using both the original hemicelluloses and the barium salts obtained after hydrolysis; the nitric acid oxidation method was also used, except in the case of fraction C 2, which was found to contain galactose.

Hemicellulose A. This fraction was hydrolysed almost entirely to xylose; a trace of arabinose was detected, probably less than 2 % of the total sugars. The hydrolysis mixture, free from acid, had $[\alpha]_D = +20°$; no hexose could be detected therein. Judging from the yield of furfuraldehyde given by the hemicellulose, since there was practically no uronic anhydride present, a small amount of hexosan was present. During the hydrolysis, an amount of dark-coloured, insoluble substance separated, equivalent to some 8–9 % of the hemicellulose. This contained 6 % of ash and gave 18 % of furfuraldehyde (on an ash-free basis).

Hemicellulose B 1. An estimation of the amount of arabinose in the hydrolysis mixture indicated the presence of about 15 % of this sugar; at most, only some 5 % of hexosan could have been present. A very small amount of mucic acid was obtained by oxidation with nitric acid; the small yield may have been due to the fact that the relatively large amount of sugar oxidation products prevented crystallisation; or another unidentified uronic acid may have been present.

Hemicellulose B 2. This fraction gave by far the lowest yield of furfuraldehyde; the hexosan content was calculated as 70 %. The total yield of the fraction was very small, and this, combined with the very low uronic anhydride content, made impossible any attempt to identify the acid constituent. It may be noted that, in this case only, the hemicelluloses prepared from different batches of grass showed markedly different rotations; this difference was further to be observed in the rotations of the hydrolysis mixtures. The 1931 preparation had $[\alpha]_D = +53°$, while that of the 1933 batch hydrolysed in a similar manner was $+17°$. The only sugars identified were galactose and arabinose; both of these are strongly dextrorotatory, so that the presence of some other sugar of low rotation was indicated. Mannose could not be detected. Judging from the yield of arabinosediphenylhydrazone given by the hydrolysis mixture some xylose was probably present, though none could be detected with certainty; the amount present however could not have accounted alone for the low rotations observed.

Hemicellulose C 1. The arabinose content of this fraction, estimated as in the case of fraction B 1, was found to be 15 %—approximately the same as in B 1. The only difference between the two fractions seemed to be in the slightly lower uronic anhydride content of this fraction. All attempts to identify the uronic acid in the hydrolysis mixture were unsuccessful.

Hemicellulose C 2. This fraction again contained much hexose, identified as galactose. Arabinose amounted to 24 % of the hydrolysate. These two sugars, with galacturonic acid, accounted for about 50 % of the hemicellulose, the remainder being xylose. Since galactose, arabinose and galacturonic acid are frequently found in association in polyuronides, the suggestion has been made that the pentose arises from the hexose by a series of mild oxidations, through the uronic acid; xylans, on the other hand, have been supposed to arise from

glucosans in a similar manner. With this in view, attempts were made to separate the xylan portion from this hemicellulose, since it seemed possible that it represented one item of a mixture, while the other three constituents were chemically combined. The methods attempted consisted in fractional precipitation, both from acid and alkaline solutions, but the furfuraldehyde, *etc.* yields of the fractions showed no significant variation from those of the original substance. On the other hand, the values obtained for the rotations of the fractions were all distinctly higher, values for $[\alpha]_D$ between $-83°$ and $-90°$ being recorded (aqueous solution, $c=0{\cdot}7$ %), so that some intramolecular re-arrangement may have taken place.

"*Combined hemicellulose.*" Preece [1931] distinguishes between "free" and "combined" hemicelluloses, the latter being defined as that fraction extracted only by hot alkali. The hemicelluloses described above were all obtained by cold extraction; from one batch however the "combined" hemicelluloses were extracted by boiling the residues after extraction with cold 4 % sodium hydroxide for 3 hours. It was found that the extract contained only a trace of hemicellulose A, fractions B and C being present in roughly equal amounts. This was somewhat unexpected, since in the isolated state fraction A is the least soluble of the hemicelluloses.

Distribution of carbohydrate fractions in various grasses.

Small samples of various grasses, grown under comparable conditions on the experimental plots at Slough, were used for a series of estimations of certain of the cell-wall and other constituents. The plots, after cutting back closely in spring (1933), were dressed with ammonium sulphate. When the blades were some 3 inches high, the plots were again cut back closely and then allowed to grow on for a further week without receiving any further ammonium sulphate. At the end of a week, the growths were again cut down, and this crop was that used for the analyses described below. (Further details of the treatment given to the experimental plots are given by Chibnall *et al.* [1933]). The samples were all rapidly dried at 98–100°, ground and sieved, the 60–90 mesh portion being used throughout. In every case, the material was re-dried in a steam-oven for 24 hours before sampling.

The following estimations were performed on each of the grasses: ash, water-soluble substances; "reducing substances" before and after hydrolysis; pectin; hemicelluloses; cellulose; xylan associated with cellulose.

1. *Ash.* Determined on portions of 0·3 to 0·5 g. of dried grass.

2. *Water-soluble substances.* 2 g. samples of dried grass were allowed to stand for 24 hours in the cold with 100 ml. of water; the extract was removed, and the residue extracted twice with 100 ml. water for 4 hours, on the steam-bath. The combined extracts were evaporated to 100 ml. Total dry matter was estimated by drying aliquot portions in the steam-oven for 48 hours.

3. *Reducing substances.* Portions of the above aqueous extract were used for estimations of free sugar (*plus* other simple reducing substances), calculated as glucose, by Bertrand's method. Similar estimations were made on portions of the extract, following hydrolysis with 1 % sulphuric acid for 40 minutes. Longer hydrolysis resulted in loss of reducing power, due possibly to destruction of fructose.

4. *Pectic substances.* 3 g. of dry material were extracted twice with 100 ml. of 0·5 % ammonium oxalate for 6 hours on the steam-bath; the residue was well washed with boiling water. Pectin was estimated in aliquot portions of the extract by the usual calcium pectate method, after preliminary precipitation with acid alcohol.

5. *Hemicelluloses.* No satisfactory standard method is yet available for the determination of this group of substances in plant tissues. In the present case they were estimated by a method

Table V. *Cell-wall constituents of various grasses.*

All figures are mean of duplicates and are calculated as % of dried grass.

Species	Date of sampling	Period of growth days	Ash %	Total water-soluble material %	Reducing substances (as glucose) %	Reducing substances after hydrolysis %	Total pectin (as Ca pectate) %	Total hemicellulose (ash- and N-free) %	Hemicellulose A (ash- and N-free) %	Hemicellulose B (ash-free) %	Hemicellulose C (ash-free) %	C and B cellulose (ash-free) (including xylan) %	Xylan in C and B cellulose %
Rough-stalked meadow grass (*Poa trivialis*)	12. vi. 33	8	9·77	29·70	3·25	11·25	0·82	16·17	5·40	—	4·30	22·18	3·15
Timothy (*Phleum pratense*)	12. vi. 33	8	8·18	23·2	2·25	6·87	1·54	17·22	—	7·10	3·60	20·83	4·32
Chewing's fescue (*Festuca rubra* var. *fallax* Hack	9. vi. 33	8	8·32	23·0	1·01	4·70	0·90	20·70	2·76	13·55	4·37	21·38	3·89
Tall fescue (*Festuca elatior*)	9. vi. 33	8	8·55	25·5	1·87	5·62	1·62	19·92	2·77	11·02	5·70	22·56	1·86
Meadow fescue (*Festuca pratensis*)	9. vi. 33	8	7·93	24·9	1·25	6·25	1·60	18·47	3·95	9·16	5·41	24·07	3·68
,, ,,	23. vi. 33	14	—	25·1	—	3·80	2·10	17·16	2·40	8·90	5·52	—	—
Italian ryegrass (*Lolium italicum*)	12. vi. 33	8	10·1	24·7	2·87	9·05	1·04	17·60	5·26	—	2·90	23·95	3·59
Crested dog's tail (*Cynosurus cristatus*)	12. vi. 33	8	9·08	24·5	3·67	13·10	1·02	16·30	—	—	3·40	23·25	3·92
Cocksfoot (*Dactylis glomerata*)	18. xi. 33	8	8·45	20·8	—	2·50	1·60	17·90	7·81	5·13	5·76	23·05	—
,,	27. vi. 33*	—	8·16	21·9	3·54	5·51	1·97	18·31	—	—	—	33·20	8·50
,,	1931*	—	6·46	19·3	3·94	5·47	1·76	18·55	—	—	—	31·64	—

* Not previously cut; growth 12 in. high.

similar to that of Preece [1931], involving actual isolation and weighing of the fractions. To obtain concordant results it was found necessary rigidly to standardise the procedure; this precaution being observed however the method seemed satisfactory.

The residues from the pectin estimations above were air-dried and then allowed to stand for 24 hours in 50 % alcohol containing 1 % NaOH, in order to remove "lignosaccharide" [Preece 1931]. The residues from this extraction were treated exhaustively with 4 % NaOH in portions of 50 ml.; two extractions were made at room temperature, for 48 hour periods, and five further extractions on a boiling water-bath for 6 hour periods; finally the residues were boiled for 1 hour under reflux with the alkali. The total extracts were made up to 400 ml. and centrifuged in order to remove a certain amount of fine suspended matter present. The following estimations were performed:

(*a*) *Total hemicellulose.* 50 ml. of the extract were treated with 10 ml. of glacial acetic acid, followed after 30 minutes by 270 ml. of 95 % alcohol; the mixture was allowed to stand for 48 hours, when the solid was collected on the centrifuge and allowed to stand for 48 hours in 50 ml. of 80 % alcohol. The centrifuging and washing were repeated, and the solid was finally filtered off on a tared alundum crucible, washed with absolute alcohol and dried in an oven at 98° overnight. Care was taken, during this filtration, that the solid collected in a small compact mass on the crucible; this being done, the small "button" of solid was readily detached when dry. The weight of solid was corrected for ash and protein content (N × 6·25), these being estimated by the micro-methods.

(*b*) *Hemicellulose A.* 100 ml. of the extract were treated with 20 ml. of glacial acetic acid and allowed to stand for 3–4 days, and the precipitate was removed on the centrifuge. (In some cases, this was difficult; a fresh extraction, *etc.* was made in such cases.) The precipitate was washed with dilute acetic acid containing a little alcohol, then transferred to 50 ml. of 80 % alcohol, filtered and dried as before. The weight was again corrected for ash and protein.

(*c*) *Hemicellulose B.* After removal of hemicellulose A, the clear liquid (neglecting the washings) was treated with 70 ml. of acetone. After standing for 48 hours, the solid was removed, washed and dried as usual. A correction was applied for ash only.

(*d*) *Hemicellulose C.* The liquid remaining after removal of fractions A and B was precipitated with 400 ml. of 95 % alcohol, and the flocculum was removed after 48 hours, washed and dried as before. The weight was corrected for ash only.

6. *Cellulose* was estimated in portions of 2 g. by the Cross and Bevan method.

7. *Xylan in cellulose.* The furfuraldehyde yield of the Cross and Bevan cellulose was determined in each case and the xylan content of the cellulose calculated therefrom. (Hydrolysis of the Cross and Bevan cellulose from cocksfoot by the method of Monier-Williams had shown the presence of xylan in this product.) The figures given in Table V are calculated back to show the amount of this xylan in the original dry grass.

Discussion.

The pectic substances of grass have been shown to be normal in type; the amount in the tissue is definitely low. With this low pectin content is associated a relatively high content of hemicelluloses, this being in accordance with the usual observations. The relative amounts of the two classes of substance are of the order met with in lignified tissues such as timber and straws, rather than in leaves. It has been considered generally that a high content of hemicellulose is associated with a prolonged period of development, or a slow rate of growth of the tissues; that such is not necessarily the case seems indicated by the observation that grass blades of only 8–10 days' growth contain some 20 % of hemicellulose. On the other hand, such figures as have been obtained seem to show that a distinct increase of cellulose and xylan associated therewith occurs with increasing age.

With regard to the individual polyuronides, the pectic substances have been shown to exist mainly in the form of pectates; the average methoxyl content of

the pectins is low. Five hemicellulose fractions have been distinguished, but how far these can be regarded as definite entities is doubtful. Two at least of the fractions (B 1 and C 1) were very similar chemically, as regards the proportions of the constituent sugars. In the case of cocksfoot, the fractions isolated from the 1931 and 1933 batches had practically the same constants except in the case of the very small fraction B 2 (Table III), so that one may conclude that the method of separation is capable of distinguishing fairly definite fractions in the case of any particular plant tissue. The variation in fraction B 2 is not explained.

Amongst the individual sugars isolated, xylose accounts for by far the greater part. Arabinose is also widely distributed amongst the various fractions, but galactose is confined to two of the hemicelluloses. No other sugar was detected among the hemicellulose hydrolysis products, but it may be remarked that a water-soluble polysaccharide, yielding fructose on hydrolysis, was found.

Of the uronic acids, only galacturonic acid was detected with certainty, in fractions B 1 and C 2; both these fractions gave also arabinose, and it is assumed that this sugar and the uronic acid have a common origin. Although xylose was present in such large amounts in all the hemicelluloses, the corresponding uronic acid (glycuronic acid) could not be detected.

Summary.

1. The pectins and hemicelluloses of cocksfoot have been studied. Five types of the latter have been distinguished, yielding on hydrolysis mixtures of xylose, arabinose and galactose together with uronic acids. Xylose accounts for the greater part of the sugars in the hydrolysis mixture.

2. Comparative analyses of certain of the cell-wall constituents of a number of different grasses are recorded.

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REFERENCES.

Baker and Pope (1900). *J. Chem. Soc.* **77**, 696.
Branfoot (1929). A critical and historical study of the pectic substances of plants, pp. 49–54; 71–74. (London.)
Chibnall, Miller, Hall and Westall (1933). *Biochem. J.* **27**, 1879.
Heidelberger and Goebel (1927). *J. Biol. Chem.* **74**, 613.
Nanji and Norman (1928). *Biochem. J.* **22**, 596.
Norman (1928). *Biochem. J.* **22**, 749.
Norris and Preece (1930). *Biochem. J.* **24**, 59.
—— and Schryver (1925). *Biochem. J.* **19**, 676.
Pollard, Chibnall and Piper (1931). *Biochem. J.* **25**, 2111.
Preece (1931). *Biochem. J.* **25**, 1304.

CXLIV. THE ASCORBIC ACID CONTENT OF CERTAIN CITROUS FRUITS AND SOME MANUFACTURED CITROUS PRODUCTS.

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The identification of vitamin C with ascorbic acid has evoked a number of papers in which titration methods for determination of the acid have been used to estimate the amount of vitamin C in natural products. Of particular interest in this respect are the papers concerned with the juice of citrous fruits [Bennett and Tarbert, 1933; Nelson and Mottern, 1933; Harris and Ray, 1933, 2; and Bessey and King, 1933], because of the general use and the consequent nutritive importance of these juices, because of the extent to which they enter into certain manufactured products, and also because the international standard preparation of vitamin C has been defined as the fresh juice of the lemon, *Citrus limonum*.

Our own attention was drawn to this problem somewhat indirectly; our results show general confirmation of the work of the investigators cited and certain other points that seem to us of special interest.

Ascorbic acid was determined by us as $C_6H_8O_6$, molecular weight 176, mainly with 2 : 6-dichlorophenolindophenol, by a method partly that of Harris and Ray [1933, 1] and partly that of Tillmans, whose method Harris and Ray modified. A standard solution of the dyestuff was run into the solution to be titrated; where this was a natural fruit juice, no addition was made to it, but solutions made by the extraction of portions of whole fruit were brought to the necessary degree of acidity with acetic acid. Originally we used a solution containing 0·1 g. of the dye per 100 ml., but later found that a 0·05 % solution, as suggested by Bessey and King [1933], gave equally good end-points with larger titres, and we therefore adopted it. 3 ml. portions of the juices were taken for the titration. The dye solution was standardised with ascorbic acid, the purity of which was itself checked by titration with standard iodine solution.

We also made use in some instances of titration with $N/100$ iodine solution. This was done for two reasons. In the first place, it was hoped that it might help to establish the presence of reducing substances other than ascorbic acid, since it is recognised that iodine is a less specific oxidising agent than 2 : 6-dichlorophenolindophenol; in the second place, we had been given, through the courtesy of Dr W. E. Baier, a number of results obtained by him and his colleagues in the laboratories of the California Fruit Growers' Exchange, where the iodine titration method had been used to follow through its various stages the large scale concentration of orange juice. Moreover, Dr Baier and his colleagues have themselves compared the two methods, and this has also been done by Bessey and King [1933]; it seemed therefore desirable to discover whether we would obtain the same correspondences or differences as other investigators.

Where iodine was used, 5 ml. of the fruit juice, or appropriate quantities of other products diluted with water, were taken for the titration; a few drops of fresh starch solution were added, and the iodine solution was run in slowly until a permanent blue colour was obtained. The end-point was fairly good, provided sufficient time was allowed for the titration.

Reproducibility of results.

We have investigated the reproducibility of results for both methods of titration. In most cases pairs of observations were made by two different observers; 33 pairs of determinations with 2 : 6-dichlorophenolindophenol gave a maximum variation between two observers of 0·02 mg. per ml. of juice (4 % of the mean value) and an average difference of 0·005 mg. per ml. (1 % of the mean value); the corresponding figures for 12 pairs of iodine determinations were 0·03 mg. (6 %) and 0·007 mg. (1·5 %). These figures apply to fresh fruit juices, but quite reasonably good agreement was also found for pairs of results obtained by either method on fruit extracts and manufactured products. It appears from these results that the 2 : 6-dichlorophenolindophenol method, which we agree with Harris and Ray in considering the most specific chemical test available for ascorbic acid, also gives slightly but appreciably more reproducible results than the iodine method.

Comparison of methods.

On comparing the two methods, it was found that orange and tangerine juices gave by the iodine method results that were, on the average, 1·5 % higher than those obtained with the dye, though in a number of instances the iodine results were the lower, the mean difference between all determinations by the two methods being, neglecting signs, 3·5 % of the mean value. Somewhat to our surprise, the values by the dye method for lemons were higher in every case than the values by the iodine method, the average difference being 2·5 % of the mean value. This is in direct contradiction to the results reported by Bessey and King [1933], who found a significant difference for oranges, the iodine results being higher, but no significant difference for lemons. As, however, comparisons were only made by us in quite a limited number of instances, we are not prepared to say more than that we think the matter requires further investigation. We are informed by Dr Baier (private communication) that he has found the results obtained on Californian Valencia oranges to be always higher by the iodine method than by the dye method.

CITROUS FRUIT JUICES.

In Table I are summarised the results obtained by us on three different species of the genus *Citrus*. The origin of each group was taken, as far as possible, from the papers in which the fruits were wrapped; the fruits themselves were all purchased from reputable dealers, but it is, in our opinion, desirable that an investigation of this sort should be carried out on a much larger scale by those engaged in research at the actual fruit-growing centres, not only because the origin and conditions of growth of the fruit can then be confidently stated, but also because the use of large numbers of fruits would enable the results to receive the necessary statistical treatment.

In spite of the obvious handicaps attached to *ad hoc* investigations of the kind conducted by us, we think the results are self-consistent and lead to certain definite conclusions, which are discussed below.

Table I.

Nature of fruit and date of purchase	Source of fruit	No. of samples	mg. ascorbic acid per ml. juice: Range of values	mg. ascorbic acid per ml. juice: Mean value	ml. of juice per fruit: Range of values	ml. of juice per fruit: Mean value	mg. ascorbic acid in juice of one fruit: Range of values	mg. ascorbic acid in juice of one fruit: Mean value
Sweet orange (8. xii. 33)	Spain, Almeria	4	0·89	0·89	100–110	105	89–98	93
Sweet orange (seedless) (21. xii. 33)	Spain, Gandia	4	0·55–0·66	0·62	65–95	79	44–62	49
Sweet orange (8. xii. 33)	Palestine, Jaffa	4	0·53–0·63	0·58	110–125	116	61–72	67
Sweet orange (8. xii. 33)	Brazil	2	0·57–0·59	0·58	70	70	40–41	41
Sweet orange (9. xii. 33)	South Africa	4	0·34–0·68	0·57	90–115	107	39–74	60
Sweet orange (seedless) (8. xii. 33)	Spain, Tabernas	3	0·50–0·59	0·54	70–75	72	35–44	39
Sweet orange (8. xii. 33)	Brazil	4	0·40–0·42	0·40	80–90	85	34–36	35
Bitter orange (21. xii. 33)	Spain, Seville	6	0·36–0·56	0·43	25–35	31	10–17	14
Bitter orange (30. i. 34)	Spain, Seville	8	0·22–0·46	0·33	15–40	25	4–18	9
All oranges		**39**	**0·22–0·89**	**0·51**	**15–125**	**67**	**4–98**	**39**
Tangerine (8. xii. 33)	Algeria	2	0·73–0·78	0·75	35–45	40	27–33	30
Tangerine (8. xii. 33)	Spain	4	0·31–0·36	0·33	30–40	35	9–13	12
Tangerine (2. i. 34)	Jamaica	3	0·10–0·20	0·15	30–36	32	3–7	5
All tangerines		**9**	**0·10–0·78**	**0·37**	**30–45**	**37**	**3–33**	**13**
Lemon (8. xii. 33)	Spain, Malaga	3	0·62–0·73	0·68	45–60	50	33–37	35
Lemon (8. xii. 33)	Sicily, Messina	4	0·62–0·77	0·68	30–35	32	20–23	22
Lemon (8. xii. 33)	Italy	4	0·54–0·78	0·66	35–40	37	19–31	25
Lemon (21. xii. 33)	Cape Colony	4	0·47–0·62	0·54	30–40	35	17–21	19
All lemons		**15**	**0·47–0·73**	**0·64**	**30–60**	**38**	**17–37**	**24**

The figures in Table I show a very considerable range of variation for all three species of fruit. Although the range is greater for oranges than for lemons, we think this should be possibly attributed to the fact that we examined a greater number and more types of oranges than of lemons; Harris and Ray [1933, 2], who examined more lemons than oranges found a greater variation in the former than in the latter.

Stability of ascorbic acid in citrous fruits.

It seemed to us possible that there might be a loss of ascorbic acid from the plucked fruit, and that the difference between the figures of Harris and Ray [1933, 2] and our own might be due to this loss, especially since Bennett and Tarbert [1933] obtained, on the whole, higher values for the juice of freshly plucked fruits. We therefore carried out some keeping tests, without any special precautions against loss, the fruit being left in the open laboratory for approximately 4 weeks. The results are shown in Table II.

Table II. *Stability of ascorbic acid in whole fruit.*

	As purchased: No. of samples	As purchased: mg. ascorbic acid per ml.	After storage for 1 month: No. of samples	After storage for 1 month: mg. ascorbic acid per ml.
Almeria oranges	4	0·89	2	0·71
Jaffa oranges	4	0·58	2	0·52
S. Africa oranges	4	0·57	2	0·53
Tabernas oranges	3	0·54	1	0·51
Brazil oranges	4	0·40	2	0·27
Seville oranges	6	0·43	3	0·37
Spanish tangerine oranges	4	0·33	2	0·19
Averages for seven types of orange	29	0·535	14	0·44
Malaga lemons	3	0·68	1	0·67
Italian lemons	4	0·66	2	0·54
Cape lemons	4	0·54	2	0·57
Averages for three types of lemon	11	0·63	5	0·59

Concentrated orange juice.

By the courtesy of Dr W. E. Baier, we have received figures for the ascorbic acid content, determined by means of both 2:6-dichlorophenolindophenol and iodine, of orange juice immediately after expression and of the corresponding final concentrate, indicating a very small loss of potency. Although it was not possible for us to get a complete biological comparison to confirm this result, we submitted a sample of the concentrated juice to the Nutrition Department of the Pharmacological Laboratories of the Pharmaceutical Society for assay by their routine method. It was there compared biologically with a sample of pure ascorbic acid. The following figures have been obtained for this sample.

	Ascorbic acid mg. per g.
A. California Fruit Growers' Exchange	
By titration	2·54
By calculation	2·65
B. Glaxo Research Laboratory	
By titration (4. i. 34)	2·55
By titration (20. iii. 34)	2·47
C. Pharmaceutical Society	
By biological assay	2·65
By titration	2·28

The "calculated" figures from Dr Baier's laboratory are based upon the figures obtained for the fresh juice and the concentration of the finished product, as calculated from the weights of the fresh and concentrated juices. The titration figures shown were all obtained by the indicator method, though Dr Baier's technique differs from ours in certain minor details.

Marmalade.

Table III shows the result of examining a number of commercial brands of marmalade, most of which were obtained on the open market and the rest from the manufacturer, and one sample of home-made marmalade, prepared according to a well-established recipe.

Table III. *Ascorbic acid content of marmalade.*

	Apparent content of peel	Ascorbic acid (mg. per g.) Indicator method	Iodine method
A	Very high	0·14	—
B	High	0·11	—
C	Medium	0·085	—
D	,,	0·085 (0·08)*	0·095
D (2nd sample)	,,	0·07 (0·07)*	0·105 (0·105)*
E	High	0·075	—
F	,,	0·07 (0·075)*	0·07
F (2nd sample)	,,	0·07	0·07
G	Low	0·065	—
H	,,	0·06	—
I (6 samples)	Jelly (orange)	<0·01	0·035 (0·035)* 1 sample only
J (6 samples)	Jelly (lemon)	<0·01	—
K (home-made)	Medium	<0·01	—

* Values in brackets by second observer.

The figures reveal at once some correlation between the apparent amount of peel present and the ascorbic acid content.

Attention may be directed to the negative results obtained with home-made marmalade, which had presumably been submitted to a lengthy process of boiling in an open vessel; in any case, the sample was approximately a year old when the test was carried out, and it is most improbable that the commercial samples had reached this age. On the other hand, the 12 samples I and J represented six different stages of maturation of the particular marmalades, and no trace of reducing power was found in any of the samples.

Orange marmalade in this country is usually made from bitter Seville oranges. We understand that quantities of fruit in the neighbourhood of 15–30 lbs. per 100 lbs. of marmalade are normally used; a simple calculation will show that only in the very richest Seville oranges does the juice contain sufficient ascorbic acid for the reducing power of the richer marmalades to be derivable from the juice alone. It seemed to us, therefore, desirable to make at any rate a preliminary survey of the distribution of vitamin C through the different parts of the orange.

Distribution of ascorbic acid in citrous fruits.

Five Seville oranges in all were examined, as well as single specimens of sweet orange, tangerine and lemon. Extracts from different portions of the outer yellow skin (flavedo), the inner thick white skin (albedo) and the pips and pith were titrated, and the results of these experiments are summarised in Table IV.

Table IV.

Ascorbic acid (mg. per g.).

Fruit	Flavedo	Albedo	Whole peel	Juice	Pips
Seville orange	2·2	0·72	1·9	0·46	<0·01
,,	1·8	0·77	0·86 (0·89)*	0·22	<0·01
,,	—	—	0·96	0·30	—
,,	—	—	0·96	0·32	—
,,	—	—	1·2	0·28	—
Sweet orange	1·3 (1·4)*	0·69 (0·68)*	1·01 (1·02)*	0·72	—
Tangerine	—	—	0·69	0·15	—
Lemon	2·1 (2·2)*	0·46 (0·51)*	1·0	1·0	—

Weights of portions.

Fruit	Flavedo g.	Albedo g.	Whole peel g.	Juice ml.	Pips g.
Seville orange (average)	10	31	41	25	30
Sweet orange	12	31	43	40	30
Tangerine	—	—	18	30	7
Lemon	—	—	20	30	20

Average distribution of ascorbic acid in whole orange (Seville) as % of total.

	%		%
Flavedo	40	Juice	15
Albedo	45	Pips	0

* Values in brackets by iodine titration.

Discussion.

The scope of our investigation naturally does not allow us to correlate the variation in ascorbic acid content of citrous fruit juices with many factors that might conceivably have a determining influence.

We have been informed that there is a considerable variation in the content of fruit solids not only in different fruits from the same tree, which might be

correlated with different degrees of ripeness, but in average fruits from neighbouring trees of undoubted genetic identity, grown under identical conditions, and there is no *a priori* reason for assuming that these variations will not apply to the ascorbic acid content.

Comparison of our results with those of Bennett and Tarbert showed that their averages for both oranges and lemons are definitely higher than ours. On the other hand, Harris and Ray found a considerably lower average value for lemons than we did. Table V summarises the figures reported by five groups of investigators for the ascorbic acid content of orange and lemon juices.

Table V.

	Oranges					Lemons				
	Maximum group range	Maximum group variation as % of mean	Total range	Maximum variation as % of mean	General mean	Maximum group range	Maximum group variation as % of mean	Total range	Maximum variation as % of mean	General mean
Bennett and Tarbert	—	—	0·91–1·03	15	0·97	0·43–0·88	70	0·43–0·94	75	0·68
Bessey and King	—	—	—	—	0·60	—	—	—	—	0·57
Harris and Ray	0·45–0·76	45	0·48–0·91	67	0·63	—	—	0·34–0·69*	73	0·47
Baier‡	—	—	0·38–0·53	33	0·45	—	—	—	—	—
Bacharach, Cook and Smith	0·34–0·68	67	0·22–0·89	130	0·51	0·54–0·78	38	0·47–0·78	50	0·63

* Omitting one extremely low value (0·18).

‡ Group values—all Valencias.

Possible causes of variation in the ascorbic acid content of orange juice are many; among them are climatic conditions, including seasonal and annual variations in sunshine, rainfall and humidity, variety of fruit, soil and other cultural conditions, size and maturity of individual fruits, method of ripening and so on.

We think the results obtained by us on fresh fruit juices establish fairly definitely the contention made by one of us recently [Bacharach, 1933] that there is a dangerous tendency to generalise on the basis of insufficient experimental evidence about the amount of a particular vitamin "naturally" present in a given foodstuff. We have obtained indications not only of a very considerable range of values of ascorbic acid in, for example, oranges of different variety and origin, but even of very marked variations between the amounts present in the juice of fruits taken at hazard from the same consignment. A certain amount of work on this subject has already been published, but it is clear that the field remains still largely virgin soil.

In contradistinction to the definite loss of ascorbic acid found by us in oranges that had been kept for 1 month, Bracewell and Zilva, using the biological test [1931], reported "no marked loss in the vitamin content [of Jaffa oranges] when the fruit is stored under ordinary conditions at about 15° for 2 months." It is, however, doubtful whether the amount of change taking place under the conditions mentioned by them would have been detected by biological assay, unless they had been able to use very much larger numbers of animals for the comparison and had been able to compare the juice before and after storage with some stable standard preparation.

We think our figures give evidence of deterioration even in so short a time as 1 month, and it seems to us a matter of some interest to those concerned with dietetics or with the fruit trade to see to what extent this change is normal and inevitable, for how long it continues, and what are the resultant limiting values for ascorbic acid content in different citrous fruit jucies. It will be remembered

that Harris and Ray [1933, 2] found an apparent increase of ascorbic acid on storing lemons and attributed it to desiccation. The two effects are clearly antagonistic and one might mask the other.

The literature contains various accounts of investigations into the anti-scorbutic potency of preserved (canned), concentrated, and dried orange and lemon juices, but in many cases the material examined was prepared on a relatively small scale. Priston [1926] described the examination of a concentrated Californian orange juice; his results suggested that there had been little destruction of its vitamin C content during processing. There were, however, no comparisons possible between the actual potency of the fresh juice and that of the manufactured product.

Goss [1925] tested biologically a number of products prepared commercially, including a vacuum-concentrated lemon juice, dried whole orange, desiccated orange juice and clarified concentrated orange juice; of the last named he reported that it "retained the antiscorbutic factor to a great extent although there is a probability that the longer exposure to the air at higher temperatures lowered this value." He used fresh orange juice as a standard antiscorbutic for control; this may have differed somewhat in vitamin C content from the fresh juice that was concentrated.

The results obtained on the sample of concentrated Californian orange juice, assayed at the Pharmacological Laboratories of the Pharmaceutical Society, indicate the possibility of conserving vitamin C even in large scale manufacturing processes. The excellent agreement between the biological and chemical assays again points to the presence in orange juice of negligible amounts of non-anti-scorbutic substances having a reducing action on the dyestuff and iodine. It may be noted that, from Dr Baier's figures for the ascorbic acid contents of the original and the concentrated juice, and the weight concentration effected, it can be calculated that about 95 % of the original ascorbic acid is retained in the final product.

We feel justified in stating on the basis of the various results reported in this paper, that the preservation of vitamin C in products such as marmalade should offer no serious difficulties in properly organised and adequately controlled manufacture. By appropriate selection of fruit containing the right quantity and distribution of vitamin C, with special reference to the proportion of peel and juice to be included in the finished marmalade, the manufacturer should be able to produce a marmalade of standardised vitamin C content, and of any value required, within the limits permitted by the natural content of the fruit. The matter is by no means one of purely academic interest; there must be many households where practically the sole sources of vitamin C available to a growing family are potatoes and marmalade; variations in the antiscorbutic potency of the latter might have an appreciable effect on the vitamin C intake of the growing child.

As far as we have been able to discover, only one biological estimation of the vitamin C in marmalade has been reported in the literature. Delf [1921] fed to a scorbutic monkey a sample of orange marmalade, made without any special precautions to prevent destruction, except that the peel and solid parts had been boiled first and the juice added at the end. Delf reported that the marmalade had "a definite but small antiscorbutic value," about 1/10th that attributed by her to fresh orange juice. Delf, like ourselves, not unnaturally assumed that the vitamin C in marmalade was derived entirely from the orange juice used.

A comparison of the antiscorbutic potency of a sample of marmalade and the juice of fresh bitter oranges similar to those from which the marmalade had

been made was carried out in 1928 in the laboratories of the Pharmaceutical Society of Great Britain [Coward, 1934, private communication]. The marmalade was found to have about one-fourth to one-third of the vitamin C potency of the bitter orange juice. As the juice was known to constitute only about one-sixth of the finished marmalade, it was evident that some other constituent was contributing to the vitamin C content. The peel of the orange was suspected on the evidence of Hess [1916] as to the presence of vitamin C in orange rind, though no further experiments were carried out to try to confirm this.

Our results seem to us to indicate that a very considerable part of the vitamin C in marmalade, where it is present to an appreciable extent, is derived from the peel rather than from the juice. There is a marked correlation between the amount of peel apparently present in the marmalade, as far as can be judged by inspection, and the amount of ascorbic acid determined by titration, in spite of the probability that there must be numerous other independent variables operating to determine the final resultant ascorbic acid. In one or two instances we obtained higher results by the iodine titration than by the 2:6-dichlorophenolindophenol method. This might conceivably be due to the production during manufacture of sugar degradation products susceptible of oxidation by iodine solution but not reducing the dye under the conditions of the test. Nor must the possibility be overlooked that there may be produced sugar degradation products capable of reducing both reagents; there is no method known at present for establishing this point, save an extensive series of comparative chemical and biological tests. The recent work of Euler and Martius [1933] and others shows that physiologically inactive reducing substances can be prepared from simple sugars by heating under alkaline conditions, but we have found it also possible to obtain an increase in the reducing power of malt extract by simply heating it for ½ to 2 hours at 100°; it is conceivable that traces of these substances might be produced during marmalade manufacture, even in the presence of natural fruit acids.

On the other hand, we have obtained some indication of the presence of inhibiting substances, or at any rate of technical difficulties in the estimation that result in the apparent presence of inhibiting substances. When pure ascorbic acid is added to orange or lemon juice, almost complete "recovery" is obtainable by either of the titration methods. This is not always the case if pure ascorbic acid is added to marmalade. Nevertheless, even bearing in mind the possible presence of reducing substances other than ascorbic acid, and of substances inhibiting the reduction of dyestuff and iodine, as well as the experimental errors inseparable from the titration of samples of marmalade, we are of opinion that the correlation between the apparent peel contents of marmalades and their reducing powers is too close to be other than significant.

Our results with the peel of citrous fruits might possibly have been attributed to the presence of reducing substances other than ascorbic acid, although the agreement between iodine and dye titrations is some evidence against such a view. We were therefore interested to find in the literature references to the antiscorbutic properties of orange peel. Hess and Fish [1914] report that "the juice of orange peel seems to serve the same purpose as the juice of the orange itself" in clinical experiments on infants. Hess and Unger [1918, 1] report that "a watery decoction of orange peel was able to protect against scurvy," and also [Hess and Unger, 1918, 2] that "orange peel possesses marked antiscorbutic potency, and withstands desiccation remarkably well." Willimott and Wokes [1926], however, found that an alcoholic extract of lemon flavedo was only feebly antiscorbutic; the conditions of preparation and storage of their

extract may well, however, have been such as to destroy any ascorbic acid present.

If our results lend themselves to the interpretation that there is a steadily falling concentration of vitamin C as one passes from the outside to the inside of the orange, it seems to us to open up some lines of investigation into the actual processes whereby ascorbic acid is synthesised in the plant. Is it possible that ascorbic acid is produced by some photosynthetic process involving the pigments themselves, and if so, which of the pigments in orange flavedo are responsible?

We shall content ourselves here with pointing to the unexpected similarity shown by the orange and the apple, since Bracewell *et al.* [1931] have fully demonstrated that the concentration of vitamin C in the tissue of Bramley's Seedling apples increases as the skin is approached from the core, and is more than six times as great in the peel as in the flesh near the core. The apparently very different morphology of the orange led us to assume, as we fancy has been assumed by other investigators, that the natural site of its vitamin C would be the juice, and that any present in the peel would be more or less adventitious. Our results suggest that this is very far from the truth.

The antiscorbutic activity of ascorbic acid has been generally accepted as about 16,000 units per g., but Key and Morgan [1933] in a recent paper reported only half this value and suggested that the discrepancy might be attributed to natural variations in lemon juice. The paper previously published by Bennett and Tarbert [1933] and the subsequent publication by Harris and Ray [1933, 2] lend some support to this suggestion, though Key and Morgan's value would necessitate that their lemon juice contained 1·35 mg. ascorbic acid per ml., a figure higher than has ever been reported by chemical determination, as indeed Harris and Ray themselves observe. The matter can never be finally settled since Key and Morgan carried out no chemical determinations of ascorbic acid on the lemon juice used for their comparisons. Qualitatively, however, we confirm the finding of Harris and Ray that fresh lemon juice as such, without further qualification, is obviously unfitted for use as an international standard preparation in vitamin testing.

The mean value found by us for lemon juice was 0·64 mg. per ml., which assigns to the international unit of vitamin C an ascorbic acid equivalent of 0·064 mg. This would give to ascorbic acid a value of nearly 16,000 international units per g. Since it is clear that our relatively small number of samples did not by any means cover the whole range of variation in lemon juices, no serious inaccuracy would be introduced into assays that have already been completed if the Permanent Commission for Biological Standardisation were to revise the international standard for vitamin C assay and assign to pure ascorbic acid an antiscorbutic activity of 10,000 or 20,000 international units per g.

Summary.

Values are presented for the ascorbic acid content of the juice of individual fruits of oranges from nine different sources, tangerines from three sources and lemons from four sources. Determinations were made by titration with 2:6-dichlorophenolindophenol in acid solution, and in many cases with iodine also. These two methods gave results in good agreement.

Thirty-nine oranges gave extreme values of 0·22 and 0·89 mg. ascorbic acid per ml. of juice, with a mean of 0·51 mg. per ml. Nine tangerines gave extreme values of 0·10 and 0·78 mg. per ml., with a mean of 0·37 mg. per ml. Fifteen lemons gave extreme values of 0·47 and 0·73 mg. per ml., with a mean of 0·64 mg.

per ml. Considerable variations were often found even between fruits from the same consignment.

Storage of the fruit for 1 month caused on the average nearly 20 % loss of ascorbic acid in oranges, but only 6 % in lemons.

Examination of a concentrated orange juice by both chemical and biological methods showed that negligible loss of ascorbic acid had occurred during concentration and subsequent storage.

Eleven varieties of marmalade contained 0·06 to 0·14 mg. ascorbic acid per g. except for three varieties, which gave a negative test. The richer kinds were those apparently containing most peel.

In all types of citrous fruits examined, the flavedo was richest in reducing power, the albedo next richest; the juice was poorer in ascorbic acid than the peel, while the pips contained no demonstrable amount.

In view of the variable antiscorbutic potency of lemon juice, it is suggested that the international unit of vitamin C should be redefined as the vitamin C activity of some definite weight of ascorbic acid.

REFERENCES.

Bacharach (1933). *J. Soc. Chem. Ind.* **52**, 68.
Bennett and Tarbert (1933). *Biochem. J.* **27**, 1294.
Bessey and King (1933). *J. Biol. Chem.* **103**, 687.
Bracewell, Kidd, West and Zilva (1931). *Biochem. J.* **25**, 138.
—— and Zilva (1931). *Biochem. J.* **25**, 1081.
Delf (1921). *Publications of South African Institute for Medical Research*, **14**, 7.
Euler and Martius (1933). *Svensk. Kem. Tidskr.* **45**, 73.
Goss (1925). *Hilgardia*, **1**, 15.
Harris and Ray (1933, 1). *Biochem. J.* **27**, 303.
—— —— (1933, 2). *Biochem. J.* **27**, 2016.
Hess (1916). *Amer. J. Dis. Child.* **12**, 152.
—— and Fish (1914). *Amer. J. Dis. Child.* **8**, 385.
—— and Unger (1918, 1). *Proc. Soc. Exp. Biol. Med.* **15**, 141.
—— —— (1918, 2). *J. Biol. Chem.* **35**, 487.
Key and Morgan (1933). *Biochem. J.* **27**, 1030.
Nelson and Mottern (1933). *J. Ind. Eng. Chem.* **25**, 216.
Priston (1926). *J. Royal Navy Med. Service*, **12**, 1.
Willimott and Wokes (1926). *Biochem. J.* **20**, 1013.

CXLV. THE CATALYSIS OF PEROXIDE OXIDATIONS BY FERROUS IONS.

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The importance of "oxygenase" systems in biological oxidations has been abundantly shown by the work of Engler and Wild [1897], Bach and Chodat [1902–1904], Manchot [1902], Gallagher [1923], Warburg [1921–1928], and Thurlow [1925]. The "oxygenase" theory may be briefly summarised as follows.

There exist in living cells certain autoxidisable substances capable of uniting with molecular oxygen and so giving rise to highly oxygenated peroxide-like compounds which act as powerful oxidising agents. If A represents such an autoxidisable substance and B a substance capable of acting as an oxygen acceptor, then the oxidation of B is represented by the following scheme:

$$A + O_2 \longrightarrow AO_2$$
$$AO_2 + 2B \longrightarrow A + 2BO.$$

A model for this reaction is to be found in the well-known induced oxidation of indigo to isatin in the presence of benzaldehyde, first described by Baeyer and Villiger [1900].

The transference of oxygen from the "peroxide" (AO_2) to the oxidisable substance B is made possible by the intervention of enzymic systems known as peroxidases. How these exert their specific influence is still a matter of conjecture, but it is thought that metals such as iron, copper and manganese may play a part. Warburg [1925], indeed, ascribes to iron an extremely important rôle in tissue oxidations and postulates a complete "oxygenase" system in which the iron activates molecular oxygen and brings about its transference to organic substances. Closely related is the *in vitro* function of iron as a catalyst in reactions between organic substances and oxygen which is already in an activated form. As such the function of iron is that of a peroxidase. This catalytic function of ferrous iron was first observed by Fenton [1898] who demonstrated the oxidation of tartaric acid to dihydroxymaleic acid by hydrogen peroxide in the presence of ferrous salts. Brode [1901] put forward the view that an unstable compound is first formed by combination of the ferrous salt and hydrogen peroxide and that this is the actual catalyst. Mummery [1913], investigating the reaction between ferrous salts and hydrogen peroxide, found that each gram-atom of ferrous iron required for oxidation to the ferric state one half-gram-molecule of hydrogen peroxide, and further, that hydrogen peroxide may be catalytically decomposed by addition to a solution of a ferrous salt. Mummery made use of Trillat's reagent (tetramethyldiaminodiphenylmethane in citric acid solution) which gives a blue colour with the lead dioxide class of superoxides but remains unchanged in the presence of true peroxides. He found that when hydrogen peroxide is added to a solution of a ferrous salt containing Trillat's

reagent a deep blue colour results. No colour is given when Trillat's reagent is added to a solution of a ferrous salt after the addition of hydrogen peroxide. The reagent is not coloured by ferrous salts, ferric salts or hydrogen peroxide alone or by ferric salts in the presence of hydrogen peroxide.

Mummery concluded from these findings that in the oxidation of ferrous salts by hydrogen peroxide there is an intermediate stage with the production of an unstable and extremely active oxidising agent. He suggested that this might be the actual oxidising agent in Fenton's reaction and assigned to it a perhydrol structure HO.O—Fe—SO_4H, ferrous sulphate perhydrol.

More recently Küchlin and Boëseken [1929], studying the oxidation of fructose and glycerol, have suggested that in such oxidation systems the first reaction is the formation of a binary complex of ferrous salt and the oxidisable substance. This may then be followed by an addition of hydrogen peroxide giving a ternary complex which is disrupted by the act of oxidation, liberating ferric iron and the oxidation product.

It is evident from the foregoing résumé that the rôle of iron in catalysed oxidations is far from clear. It is still uncertain whether the iron undergoes a change of valency while acting as a catalyst and how such a change of valency, if it does take place, is related to other reactions occurring between members of the system. The present study has been directed on both qualitative and quantitative lines towards the elucidation of the reactions involved in the catalysis of peroxide oxidations by means of ferrous iron. The qualitative observations are reported in Part I and the quantitative aspects in Part II.

I. Qualitative aspects.

Several systems of the form hydrogen peroxide—ferrous iron—oxidisable substance have been examined. Qualitative tests have been carried out to determine the fate of each member of the system, while any special features of each system having a bearing on the points at issue have been noted.

Ferrous iron was detected by means of the ferricyanide reaction and ferric iron by the colour given with thiocyanates. Hydrogen peroxide was detected by means of the blue ether-extractable colour given with chromic acid. Oxidisable substances have been used in the form of stock solutions of known strength. Dilutions were made from these as required. Ferrous salts have been used in the form of freshly prepared solutions of known strength in glass-distilled CO_2-free water. Hydrogen peroxide was used in the form of neutral dilutions from Merck's perhydrol. Each solution was standardised against $KMnO_4$ immediately before use.

The method of carrying out the examinations was as follows:

A measured quantity of the stock solution of the oxidisable substance was added to a known volume of the ferrous salt solution. This was followed by distilled water of such volume that after addition of the hydrogen peroxide solution (which was added last) the required concentrations of the reagents were attained.

After addition of the hydrogen peroxide the qualitative tests were carried out.

The oxidation of tartaric acid.

The following solutions were examined:

(1) 0·1 *M* tartaric acid which was 0·01 *M* with respect to ferrous salt and 0·01 *M* with respect to hydrogen peroxide.

(2) 0·1 *M* tartaric acid which was 0·01 *M* with respect to ferrous salt and 0·1 *M* with respect to hydrogen peroxide.

(3) and (4) as (1) and (2) but without tartaric acid.

Controls:	1	2	3	4
Hydrogen peroxide	−	−	−	+
Ferrous iron	+	+	−	−

In solutions 3 and 4 addition of peroxide gave rise to a strong orange-yellow colour which persisted—the thiocyanate test being strongly positive.

Addition of hydrogen peroxide to solutions 1 and 2 gave a greenish yellow colour which faded rapidly giving a water-clear solution free from ferric iron. A positive thiocyanate reaction was only given while the greenish yellow colour persisted in the solution.

It was noted that the time required for the disappearance of the colour produced by addition of hydrogen peroxide to a solution of tartaric acid containing ferrous iron became less with successive additions.

A typical example may be given.

0·1 *M* tartaric acid containing 0·01 *M* ferrous sulphate was taken.

0·01 *M* hydrogen peroxide was added and the time taken for the colour so produced to fade was noted by means of a stop-watch. A second proportion of peroxide was now added and the time again noted, the process being repeated until, in all, ten additions of peroxide had been made.

No. of additions of peroxide	1	2	3	4	5	6	7	8	9	10
Time in seconds	194	130	83	81	79	75	76	85	74	74
	198	128	81	81	78	76	74	72	73	72

It will be seen that the time required for the disappearance of the ferric iron initially formed decreases as the concentration of the tartaric acid decreases—the reduction therefore cannot be brought about by a reaction in which tartaric acid plays a part.

To a solution of tartaric acid containing ferrous iron there were added a few drops of Trillat's reagent (1 % tetramethyldiaminodiphenylmethane in 1 % citric acid). On addition of hydrogen peroxide a deep blue colour was formed. This, however, quickly faded giving a water-clear solution.

A solution of tartaric acid containing ferrous iron was divided into two portions *a* and *b*. To *a* a few drops of Trillat's reagent were added. Hydrogen peroxide was then added to both solutions. It was observed that the colours initially formed faded rapidly, the blue of *a* and the greenish yellow of *b*, and that the solutions became colourless simultaneously. No colour was given on the addition of Trillat's reagent to solution *b* after the fading of the greenish yellow colour. Addition of Trillat's reagent while the greenish yellow colour persisted gave the blue colour.

The above observations lead to the conclusion that the same substance is responsible for giving the ferric iron test, the greenish yellow colour of the solution and the reaction with Trillat's reagent. It appears to have but a temporary existence, and the rate of its disappearance does not appear to bear any relationship to the amount of tartaric acid present in the solution.

A further experiment bearing on the production of this labile substance may be reported.

To a solution of a ferrous salt an amount of hydrogen peroxide more than

sufficient to oxidise all the iron to the ferric state was added. Immediately the solution became brown in colour and effervescence took place. If allowed to stand the effervescence stopped and a brown precipitate formed. No ferrous iron could be detected in the solution.

If, however, a solution of tartaric acid were added while effervescence was taking place the deep brown colour of the solution faded immediately to greenish yellow. This latter colour persisted for some time and finally faded to leave a water-clear solution. Only ferrous iron could now be detected in the solution. The production of the labile substance mentioned above does not seem therefore to be conditional on the presence of ferrous iron in the solution.

The discussion of the facts reported in this section is postponed until the examination of other oxidation systems has been described. The reactions taking place during the oxidation of tartaric acid appear to proceed very rapidly and it was felt that it would be advantageous to examine systems requiring a longer time for the completion of the various phases of reaction.

The oxidation of d-*glucose.*

d-Glucose was shown to be oxidised to glucosone by Fenton's reaction by Morrell and Crofts [1899].

Preliminary study revealed that with *d*-glucose the reaction appeared to proceed at a much slower rate than with tartaric acid, and it has accordingly been chosen to illustrate certain points.

When hydrogen peroxide is added to a solution of *d*-glucose containing ferrous iron there is initially developed a yellowish brown colour. This, as shown by a positive thiocyanate test, is due to the presence of ferric iron. The colour persists for a long time but slowly disappears until finally a solution is obtained which is only slightly yellow in colour. This solution is now free from ferric iron but contains ferrous iron.

It was found that on adding sufficient hydrogen peroxide the presence of this reagent could be detected in solution. The peroxide was found to disappear quite rapidly from the solution, the time required depending on the amount of peroxide added. The disappearance of the ferric iron did not, however, coincide with the disappearance of the peroxide. Indeed a very much longer time was required for the solution to react negatively to thiocyanate. In one experiment a solution was made 0·1 *M* with respect to *d*-glucose, 0·01 *M* with respect to ferrous sulphate and 0·1 *M* with respect to hydrogen peroxide, the oxidising agent being added last. It was found that all the peroxide had disappeared from the solution in 12 minutes, while the ferric iron did not disappear until after 24 hours had elapsed.

Table I.

d-Glucose 0·1 *M* and $FeSO_4$ 0·01 *M* throughout.

No. of tube	1	2	3	4	5	6	7	8	9	10
Molarity hydrogen peroxide	0·01	0·02	0·03	0·04	0·05	0·06	0·07	0·08	0·09	0·10
Ferric iron	+	+	+	+	+	+	+	+	+	+
Ferrous iron	+	+	+	+	−	−	−	−	−	−
Hydrogen peroxide	−	−	−	−	+	+	+	+	+	+

In Table I are given the results of observations on a typical glucose system, the tests being carried out immediately after the addition of hydrogen peroxide.

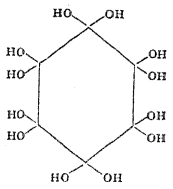
It will be seen that in every case, with additions of peroxide up to concentrations representing 4 g.-mol. proportions of hydrogen peroxide per g. atom of iron, ferric and ferrous iron were found in the solution. The intensity of the thiocyanate test increased as that of the ferricyanide test decreased. The presence of hydrogen peroxide could be detected only in those tubes from which all the ferrous iron had disappeared. Solutions 5–10 were of a uniform yellow colour; all showed the presence of hydrogen peroxide and absence of ferrous iron.

The difference in times taken for the disappearance of ferric iron and hydrogen peroxide strengthens the impression obtained in the study of tartaric acid oxidations that the reappearance of ferrous iron is not associated with the primary oxidation taking place in such systems. The amount of hydrogen peroxide required to be added before all the ferrous iron disappears should be contrasted with the 0·5 g.-mol. proportion required in the absence of an oxidisable substance. This point is dealt with more fully in Part II.

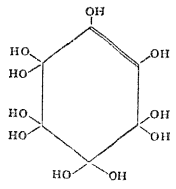
The oxidation of i-*inositol.*

So far no information has been obtained as to the behaviour of the oxidisable substance in such systems. In order to provide this, some observations may be reported on the properties of peroxide-iron systems containing *i*-inositol.

In the course of a study of the oxidation of *i*-inositol it was observed that addition of hydrogen peroxide to solutions of *i*-inositol containing ferrous iron gave rise to the production of a deep blue colour. This colour is the result of a reaction between ferric ions and the first oxidation product of *i*-inositol—an example of the general reaction between ferric ions and substances containing phenolic groups.



i-Inositol



First oxidation product
(Gives blue colour with ferric ions)

It will be readily appreciated that such systems are self-indicating so far as the presence of oxidation product is concerned.

When hydrogen peroxide is added to a solution of *i*-inositol containing ferrous iron there is initially developed a yellowish brown colour and the thiocyanate test is positive. In a short time the yellowish colour merges into green and finally into a deep blue. This colour persists for a considerable time but gradually fades leaving a water-clear solution giving no reaction for ferric iron.

In Table II is given a summary of a typical experiment with *i*-inositol.

In tubes 1–4 there was no development of blue colour, showing that no oxidation of the *i*-inositol had taken place. In these tubes the ferric iron did not disappear on standing.

In tubes 5–10 the development of the blue colour was followed by a gradual fading to a water-clear solution free from ferric iron.

Table II.

i-Inositol 0·1 *M* and $FeSO_4$ 0·01 *M* throughout.

No. of tube	1	2	3	4	5	6	7	8	9	10
Molarity hydrogen peroxide	0·01	0·02	0·03	0·04	0·05	0·06	0·07	0·08	0·09	0·10
Ferric iron	+	+	+	+	+	+	+	+	+	+
Ferrous iron	+	+	+	+	−	−	−	−	−	−
Hydrogen peroxide	−	−	−	−	+	+	+	+	+	+
Development of blue colour	−	−	−	−	+	+	+	+	+	+

There would not appear to be any oxidation of the *i*-inositol until all the iron is converted into the ferric state.

The oxidation of formic acid.

The substances examined so far, tartaric acid, *d*-glucose and *i*-inositol, yield on oxidation products which are themselves capable of further oxidation, thus introducing secondary effects. In order to rule out such secondary effects it was decided to study the behaviour of formic acid. This substance gives on oxidation carbon dioxide and water which, being incapable of further degradation, do not introduce secondary effects.

Table III summarises a series of experiments on the oxidation of formic acid.

Table III.

Formic acid 0·1 *M* and $FeSO_4$ 0·01 *M* throughout.

Tube No.	1	2	3	4	5	6	7	8	9	10
Molarity hydrogen peroxide	0·01	0·02	0·03	0·04	0·05	0·06	0·07	0·08	0·09	0·10
Ferric iron	+	+	+	+	+	+	+	+	+	+
Ferrous iron	+	+	+	+	−	−	−	−	−	−
Hydrogen peroxide	−	−	−	−	+	+	+	+	+	+

This system corresponds exactly with those already reported with the exception that although the hydrogen peroxide disappears rapidly there is no regeneration of ferrous iron. Instead, on standing, the solutions deposit a brownish yellow precipitate which is soluble in mineral acids and gives a positive test for ferric iron.

II. Quantitative aspects.

Mummery [1913] has found by means of qualitative tests that in the presence of sulphuric acid, 1 g.-mol. of ferrous iron requires for its oxidation to the ferric state 0·5 g.-mol. of hydrogen peroxide.

$$2FeSO_4 + H_2O_2 + H_2SO_4 = Fe_2(SO_4)_3 + 2H_2O.$$

In the present study ferrous iron is oxidised by hydrogen peroxide and it was desirable to find the quantitative relationships existing between these two substances under the conditions employed.

Procedure. Tubes were made up, each containing a known quantity of ferrous sulphate solution. Known quantities of hydrogen peroxide in increasing amounts throughout the series were then added. The solutions were immediately acidified with sulphuric acid and titrated with potassium permanganate solution. The results of two such experiments are shown in Table IV.

It will be seen that the permanganate titre is least in tube 7 and it may be concluded that in the absence of sulphuric acid 1 g.-mol. of hydrogen peroxide

Table IV.

Tube No.	1	2	3	4	5	6	7
g.-mol. ferrous salt	0·001	0·001	0·001	0·001	0·001	0·001	0·001
g.-mol. peroxide/ferrous salt	—	0·1	0·2	0·3	0·4	0·5	0·6
ml. 0·04868 N $KMnO_4$	19·8	15·8	12·0	8·9	5·1	2·5	0·3
	19·8	15·9	12·0	8·9	5·3	2·3	0·2

oxidises 2 g.-mol. of ferrous salt to the ferric state. The somewhat higher figure obtained in the absence of sulphuric acid is due to catalytic decomposition of some hydrogen peroxide.

The results of Part I already reported indicated that secondary reactions in the system chosen for quantitative study would give rise to complications, and in fact it was found that the only oxidisable substance whose oxidation could be followed simply and quantitatively was formic acid.

The influence of the ratio formic acid/ferrous salt on the amount of iron oxidised.

In the qualitative study of the oxidation of formic acid it was found that there was a simultaneous oxidation of ferrous iron. Reference to Table III shows that in the conditions there represented before all the iron is converted into the ferric state there must be in the system an initial concentration of 5 g.-mol. of hydrogen peroxide per g.-atom of iron. This figure was by no means constant—appearing to depend on the ratio formic acid/ferrous salt, as is shown in the following experimental series.

For each ratio ten tubes were made up, each containing the same amounts of formic acid solution and ferrous sulphate solution. Distilled water was then added so that after addition of hydrogen peroxide each tube would contain the same volume of liquid. Hydrogen peroxide solution was added finally and in increasing amount from tube 1 to tube 10. The contents of the tubes were immediately acidified with sulphuric acid, and the amount of ferrous iron remaining in each was determined by permanganate titration. Figs. 1 to 5 show the results in graphical form. In Exps. 2 and 3 (Figs. 2 and 3), solutions of a duplicate series were not acidified with sulphuric acid immediately after the addition of hydrogen peroxide, but were allowed to stand closely stoppered for 17 hours, in which time a brown precipitate settled out. They were then acidified and titrated with permanganate solution.

It will be seen that ferric iron appears in the system long before all the formic acid can have been oxidised. The oxidation of iron and formic acid is therefore simultaneous, or at least the oxidation of the former is not brought about by excess peroxide after all the formic acid has been oxidised. Moreover, the greater the amount of hydrogen peroxide in the system, the greater the amount of iron oxidised. The sharp angle of the curves after meeting the abscissa is due to the presence of excess peroxide in those solutions which were titrated immediately. It will also be seen that it is possible to have all the iron oxidised to the ferric state before sufficient hydrogen peroxide has been added to bring about the complete oxidation of all the formic acid. Although the amount of hydrogen peroxide which must be added initially before all the ferrous salt is oxidised varies greatly with the ratio formic acid/ferrous salt, Fig. 4 shows that this amount is not dependent on the absolute concentrations of the members of the system. With a high ratio formic acid/ferrous salt a larger amount of hydrogen

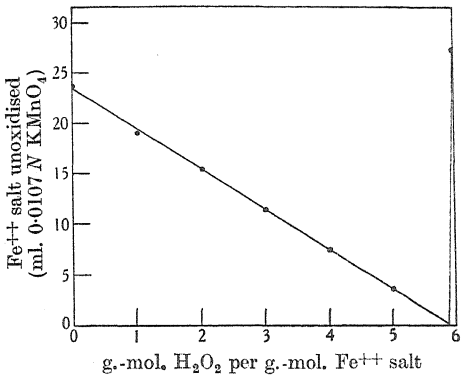


Fig 1 $\frac{\text{Formic acid}}{\text{Ferrous salt}} = \frac{20}{1}$.

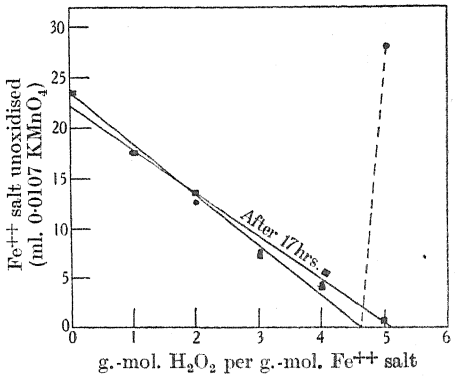


Fig. 2. $\frac{\text{Formic acid}}{\text{Ferrous salt}} = \frac{10}{1}$.

• Acidified immediately; ■ acidified after 17 hrs.

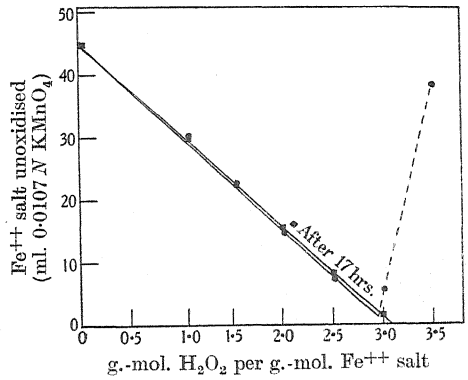


Fig. 3. $\frac{\text{Formic acid}}{\text{Ferrous salt}} = \frac{5}{1}$.

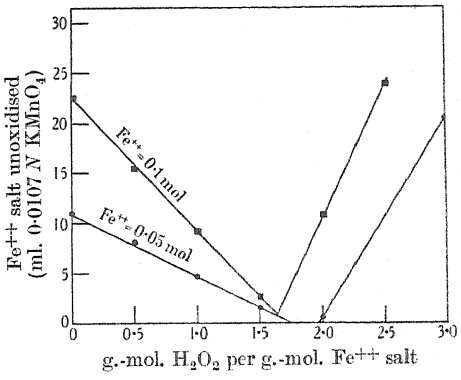


Fig. 4. $\frac{\text{Formic acid}}{\text{Ferrous salt}} = \frac{2}{1}$.

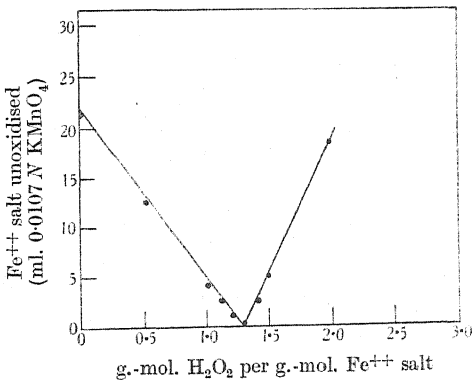


Fig. 5. $\frac{\text{Formic acid}}{\text{Ferrous salt}} = \frac{1}{1}$.

peroxide must be added to bring about oxidation of ferrous iron than with a low one. These results are roughly summarised in Table V (Fig. 6).

Table V.

Ratio formic acid/ferrous salt	20	10	5	2	1
g.-mol. H_2O_2 which must be added initially to oxidise 1 g.-atom ferrous iron	5·85	4·8	3·0	1·6	1·4

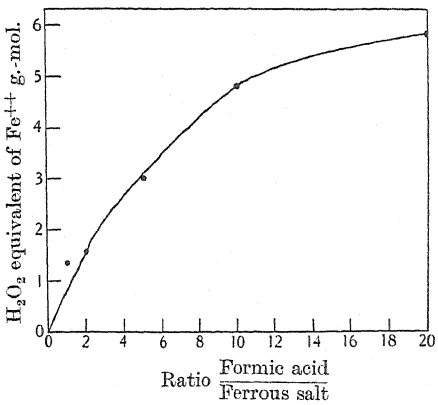


Fig. 6.

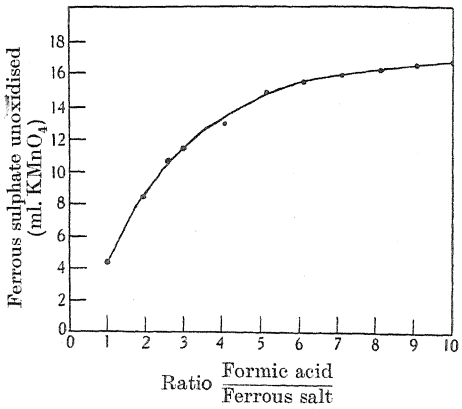


Fig. 7.

In the first series of experiments the concentrations of ferrous sulphate and formic acid were unchanged and in every case all the ferrous iron could be oxidised. The influence of the ratio formic acid/ferrous salt is also seen in the following series where the concentrations of hydrogen peroxide and ferrous salt are kept unchanged in 10 tubes while the concentration of formic acid is varied.

Table VI. (Fig. 7.)

$FeSO_4$ and H_2O_2 9×10^{-4} M throughout.

Ratio formic acid/ferrous salt	1	2	3	4	5	6	7	8	9	10
ml. permanganate solution	4·4	9·1	11·5	13·1	14·8	15·7	16	16·4	16·8	17·1

The further significance of the influence of this ratio will be considered later. Turning now to the correlation of these results with the oxidation of formic acid, the following experiments report the results of determinations of the amount of formic acid oxidised under differing conditions.

The oxidation was followed by the use of a standard baryta apparatus for the absorption of the carbon dioxide evolved. The solution containing the formic acid and ferrous salt was placed in a flask fitted with a three-holed rubber stopper carrying (1) a reflux condenser attached to a baryta absorption apparatus, (2) a burette for the introduction of hydrogen peroxide, (3) an inlet tube for CO_2-free air. The apparatus was made air-tight and free from carbon dioxide and the absorption apparatus connected. Hydrogen peroxide was introduced and the apparatus shaken for a short time. Concentrated sulphuric acid was then added, and the contents of the flask were boiled while a stream of CO_2-free air was aspirated through the solution. The amount of carbon dioxide evolved was determined by titrating the excess of baryta with standard oxalic acid solution.

It was found that (1) carbon dioxide was evolved in detectable amounts when very small quantities of hydrogen peroxide were added, (2) oxidation was inhibited by the previous addition of strong mineral acid, (3) when the ferrous salt was replaced by a normal ferric salt no oxidation took place on addition of H_2O_2, (4) if formic acid was added to a solution of a ferric salt obtained by the oxidation of a ferrous salt by the calculated amount of hydrogen peroxide, evolution of carbon dioxide followed a further addition of hydrogen peroxide.

The following table gives the results of the examination of a typical system.

Table VII.

Ratio formic acid/ferrous salt = 1. Molarity of $FeSO_4$ = 0·004.

Ratio hydrogen peroxide/H.COOH	0·5	1	1·5	2	2·5
g.-mol. hydrogen peroxide present	0·002	0·004	0·006	0·008	0·01
ml. 0·3922 *N* baryta required	5·08 5·18	9·87 10·07	15·21 15·16	18·84 18·44	20·00 19·68
g.-mol. formic acid oxidised	0·00100 0·00102	0·00197 0·00194	0·00298 0·00297	0·00369 0·00362	0·00392 0·00386
Mean	0·00101	0·00195	0·00298	0·00365	0·00389

It will be seen that the amount of formic acid oxidised is very far from being equivalent to the amount of hydrogen peroxide added. Some of the peroxide can, of course, be accounted for by the oxidation of iron which is in this case irreversible. A further series of experiments is summarised in Table VIII. In

Table VIII.

g.-mol. formic acid present	g.-mol. hydrogen peroxide present	g.-mol. ferrous salt present	ml. 0·3922 *N* baryta required to absorb CO_2	g.-mol. formic acid oxidised: Ferrous previously oxidised	g.-mol. formic acid oxidised: Ferrous not previously oxidised
0·004	0·0064	0·004	12·39 15·92	0·00243	0·00312
			12·89 16·62	0·00253	0·00326
			Mean	0·00248	0·00319
0·004	0·0084	0·004	15·02 18·76	0·00295	0·00368
			14·55 19·61	0·00285	0·00385
			Mean	0·00290	0·00376

these the oxidation of formic acid was carried out in the presence of iron previously oxidised by the requisite amount of hydrogen peroxide (0·6 g.-mol. proportion). These experiments were controlled by others in which the iron was not oxidised before the addition of formic acid.

It appears that the oxidation of formic acid by hydrogen peroxide can be catalysed by the previously oxidised iron salt. The discrepancy between the two methods was however always found. Since hydrogen peroxide disappeared from both types of system in the same way and since the amount of carbon dioxide evolved could not be increased in the first system by a longer period of aeration, the differences observed are not due to differing rates of reaction but to a smaller amount of the formic acid present actually undergoing oxidation in the first system. This is believed to be accounted for by the increased catalytic decomposition of hydrogen peroxide in the presence of the previously oxidised iron salt.

If formic acid was introduced into a solution containing initially a ferrous salt and a large excess of hydrogen peroxide, *i.e.* a solution in which the peroxide itself was undergoing catalytic decomposition, complete oxidation of the acid could be obtained provided the excess of peroxide was sufficiently large.

Table IX.

g.-mol. formic acid present	g.-mol. ferrous salt present	g.-mol. H_2O_2 present	ml. 0·3922 *N* baryta required	g.-mol. formic acid oxidised
0·004	0·004	0·04	19·56	0·00384
			19·77	0·00388
0·004	0·004	0·08	19·55	0·00393
			20·02	0·00392

In all the experiments reported the formic acid/ferrous salt ratio has been unity. It has been shown that with this ratio the amount of iron undergoing oxidation is large and that as the ratio increases, the amount becomes progressively smaller. The ratio was now varied, keeping the total volume of the solution and the amount of ferrous salt present constant and the oxidation followed as before. The results of Table X show that as the ratio increases the amount of formic acid oxidised also increases.

Table X.

Molarity of $FeSO_4$ and H_2O_2 = 0·004.

Ratio formic acid/ferrous salt	1	2	5	10	20
g.-mol. formic acid present	0·004	0·008	0·02	0·04	0·08
g.-mol. formic acid oxidised	0·00197	0·0021	0·003	0·0033	0·00343
	0·00194	0·0027	0·00293	0·00324	0·00353
Mean	0·00196	0·0024	0·00296	0·00327	0·00348
g.-mol. ferrous salt oxidised	0·0032 (*a*)	0·0023 (*b*)	0·0014 (*c*)	0·001 (*d*)	0·0008 (*e*)
g.-mol. H_2O_2 to oxidise 1 g.-atom ferrous iron	0·63	0·69	0·76	0·673	0·62

In Table XI the quantitative results obtained are correlated.

Table XI.

Ratio formic acid: ferrous salt = 1. Molarity of $FeSO_4$ = 0·004.

g.-mol. hydrogen peroxide present	0·002	0·004	0·006	0·008	0·01
g.-mol. formic acid oxidised	0·001	0·002	0·003	0·0036	0·0039
g.-mol. ferrous salt oxidised	0·00165	0·0032	0·004	0·004	0·004
g.-mol. H_2O_2 to oxidise 1 g.-atom ferrous iron	0·61	0·62	0·75	1·1	1·5

The excess hydrogen peroxide over that utilised by the formic acid is expressed in line 4, Table XI, as the amount necessary to oxidise 1 g.-atom of ferrous iron under these conditions. From a scrutiny of the table it will be seen that in this case, where the amounts of hydrogen peroxide and formic acid are molar equivalents, oxidation of the salt takes place to a greater extent than does that of the acid. The figure 0·6 g.-mol. of hydrogen peroxide for the oxidation of 1 g.-mol. of ferrous salt is obtained in Exps. 1 and 2 but not in the others. In these experiments, as the qualitative results of Table III show, there is for a short time in the solution free hydrogen peroxide. The high figures of Exps. 3, 4 and 5 can then be accounted for by catalytic decomposition of hydrogen peroxide by iron salts. The data of Table IX also support this view.

Lines 5 and 6, Table X, are compiled from the data for other values of the ratio formic acid/ferrous salt.

The figures in line 5, Table X, are calculated from the permanganate titrations reported earlier in the paper. Beside each number is given the figure from which it has been taken: (*a*) Fig. 5; (*b*) Fig. 4; (*c*) Fig. 3; (*d*) Fig. 2; (*e*) Fig. 1.

It will be seen that where all the ferrous salt has not been oxidised there is good agreement between the amount of hydrogen peroxide added to the solution and the amount equivalent to the formic acid and ferrous salt oxidised. The amount of formic acid oxidised is also seen to depend on the ratio formic acid/ferrous salt.

Discussion.

Each oxidation system which has been examined is characterised by the appearance of ferric iron. This may result in one of three ways:

(1) The change of valency of the iron may be in no way related to the catalysis.

(2) The actual catalyst may be a compound of bivalent iron, but the metal may be liberated from the catalytic reaction in the ferric form.

(3) The production of ferric iron may be a necessary preliminary to catalysis, and the catalyst may be actually a derivative of ferric iron.

The evidence is definitely in favour of the third of these possibilities.

(1) It has been shown that when tartaric acid is added to a solution of a ferrous salt containing hydrogen peroxide in amount more than sufficient for the complete oxidation of the iron, the same reaction takes place as when hydrogen peroxide is added to a solution of tartaric acid containing ferrous iron.

(2) It has been shown that when hydrogen peroxide in amount more than sufficient to oxidise all the iron is added to a solution of *i*-inositol containing ferrous iron, oxidation of the *i*-inositol takes place.

It is difficult to reconcile these two facts with the idea either that the catalyst is a ferrous iron compound liberating ferric iron after oxidation of the organic substance has taken place, or that oxidation and the appearance of ferric iron are unrelated phenomena.

It would appear that change of valency of the iron must be regarded as a necessary preliminary to the oxidation of organic substances by hydrogen peroxide when ferrous salts act in a catalytic capacity. In such systems it seems therefore that the first reaction is between the ferrous iron and hydrogen peroxide.

We have now to consider what further reactions are entered into by the ferric iron derivative, evidence for the formation of which has been reviewed above. At first sight it might seem as if we must assume the intervention of a reaction which Warburg has postulated to explain iron catalysis in the living cell, namely

$$Fe^{+++} + A \xrightarrow{\text{Oxygen}} Fe^{++} + AO$$

where A is an organic oxidisable substance.

Such a theory would require that at the end of the catalytic reaction all the iron should be in the ferrous state, for we can only conceive of the various members of the system reacting together in stoichiometrical proportions. Accordingly when the last equivalent of hydrogen peroxide in the system has raised the iron to the ferric state the reaction between the ferric compound so formed and the oxidisable substance would bring about complete reduction of the iron.

It has been observed, however, that regeneration of ferrous iron, does not take place in the case of the oxidation of formic acid, that is, with a substance

which on oxidation gives rise to products which are not further oxidisable (see Figs. 2 and 3 after 17 hours). The reduction to the ferrous state does not seem to be necessary for oxidation to take place. This conclusion is supported by further evidence. With tartaric acid systems it was shown that with subsequent additions of peroxide the rate of disappearance of ferric salt is greatly increased, apparently reaching a maximum. This cannot be explained on the basis of a reaction between ferric iron and tartaric acid, for with each subsequent addition of hydrogen peroxide the concentration of tartaric acid becomes less. If the reaction were between ferric iron and tartaric acid, according to the law of mass action, the rate of disappearance of ferric iron should decrease with each addition of hydrogen peroxide. The regeneration of ferrous iron must therefore be due to a reaction between ferric iron and a component of the system, the concentration of which increases with each addition of hydrogen peroxide. The reduction of the ferric iron must be brought about by reaction with dihydroxy-maleic acid, the oxidation product of tartaric acid.

That the reduction of the ferric iron is a secondary reaction is also shown by the fact that in the oxidation of *i*-inositol and glucose the disappearance of the ferric iron from the system is a very slow process as compared with the disappearance of hydrogen peroxide.

We are forced therefore to conclude that the ferric compound initially formed by the interaction of ferrous iron and hydrogen peroxide possesses the power of donating oxygen to oxidisable substances and that in this process there is no reversal of the initial change of valency of the iron.

Discussing now the partition of hydrogen peroxide between oxidisable substance and iron, reference to Table VI shows that as the concentration of oxidisable substance initially present in the system is increased the amount of ferrous salt finally in the system also increases. The amount of oxidisable substance undergoing oxidation correspondingly increases. It has also been shown that irrespective of the value of the ratio formic acid/ferrous salt all the iron in the system may be oxidised to the ferric state—the amount of hydrogen peroxide required varying directly with the ratio. If the theory of the catalytic function of iron presented is correct it should be possible to explain these facts in terms of it.

If the first reaction in such a system gives rise to a basic ferric salt (reaction *a*) and this salt unites with hydrogen peroxide to give an unstable iron compound which is the actual oxidising agent, then in the reaction between oxidising agent and oxidisable substance the basic ferric salt would again be produced (reaction *b*). There are then two sources of the basic ferric salt and the amount formed will depend on the amount of ferrous salt reacting with hydrogen peroxide and on the relative velocities of the reactions *a* and *b*. Any factor which would increase the rate of reaction *b* while leaving *a* unchanged would favour the production of basic ferric salt from the oxidising agent. This would then be available for further reaction with hydrogen peroxide. Such a factor would therefore tend to decrease the amount of ferrous sulphate oxidised. According to the law of mass action it is probable, therefore, that an increase in the concentration of oxidisable substance by hastening its reaction with the oxidising agent, would decrease the amount of ferrous salt undergoing oxidation.

This agrees with the known facts. It has been shown experimentally that the greater the amount of oxidisable substance in the system, the smaller the amount of ferrous salt undergoing oxidation and the greater the amount of hydrogen peroxide available for the oxidation of formic acid. It should be noted that this theory depends on a difference of velocity between the oxida-

tions of Fe^{++} salt and of formic acid which should be capable of experimental demonstration.

There is evidence that association between oxidisable substance and catalyst takes place in the systems examined. It has been noted that when hydrogen peroxide is added to solutions of ferrous sulphate there is precipitation of a brown solid which is doubtless a basic ferric sulphate produced by hydrolytic decomposition of the first product of the reaction between ferrous iron and hydrogen peroxide. This precipitation was also observed during the oxidation of formic acid. In the oxidation of tartaric acid, *d*-glucose and *i*-inositol no such precipitation was observed. Instead there was noted the reduction of the iron to the ferrous state, a process which in the case of *d*-glucose and *i*-inositol was found to be very slow. When we remember the susceptibility of the ferric derivative to hydrolytic change when no oxidisable substance is present, we can only assume that protection after the oxidation of organic substances is afforded by association with the substrate. Such association would in all probability be achieved through the free hydroxyl groups of the oxidisable substance, an assumption which might explain the precipitation observed in the oxidation of formic acid. The observations reported here as evidence of association between catalyst and substrate would be in line with the suggestion of Küchlin and Boëseken that the formation of ternary complexes is an accompaniment of the oxidation of organic substances by hydrogen peroxide in the presence of ferrous salts. It should be noted that, if such complexes are formed, the observations reported in this paper require that they should be capable of ionisation, for the thiocyanate reaction is not given by iron in a non-ionised association.

In conclusion it may be noted that the oxidation of the substrate in these systems is by no means quantitative with respect to the H_2O_2 since simultaneous oxidation of iron takes place and there is also some catalytic decomposition of hydrogen peroxide. These losses may be diminished by maintaining a high ratio substrate/catalyst.

A further source of loss is found in the reduction of iron in secondary reactions with the initial products necessitating a subsequent oxidation to the ferric state by further hydrogen peroxide. The method as used by Fenton, Morrell and Crofts, and others, depending on their own theory of the mode of the reaction, does not give the maximum amount of oxidation products. Oxidation is most favourably carried out by adding the requisite quantity of hydrogen peroxide to a solution of the substrate containing a trace of ferrous salt. When the solution gives a negative test for peroxide very little of the starting material remains unoxidised and the amount of secondary oxidation products is minimum.

Summary.

In the oxidation of organic substances by hydrogen peroxide in the presence of ferrous iron the first reaction is between the iron and the peroxide.

A derivative of ferric iron is produced and this acts as an oxygen donator.

In the reaction between this ferric derivative and the oxidisable substance there is no reduction of the iron.

The reduction of the iron is a secondary process resulting from a reaction between oxidation products and the ferric compound liberated from the reaction between oxygen donator and oxidisable substance.

There is evidence that in the catalytic oxidations considered in this paper there is formation of complexes of oxygen donator *plus* oxidisable substance.

This work was carried out by the late Dr Stirling during the tenure of the Barbour Research Scholarship in the Institute of Physiology of the University of Glasgow, and was the subject of a Thesis for the degree of Doctor of Philosophy. It was preliminary to a thorough study of the oxidation products of *i*-inositol, and it was Dr Stirling's intention to pursue this study in the quantitative manner of Part II. The quantitative studies were, however, incomplete at the time of his death on February 12th, 1933.

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REFERENCES.

Bach and Chodat (1902–1904). Papers in *Ber. deutsch. chem. Ges.*
Baeyer and Villiger (1900). *Ber. deutsch. chem. Ges.* **33**, 1569.
Brode (1901). *Z. physikal. Chem.* **37**, 257.
Engler and Wild (1897). *Ber. deutsch. chem. Ges.* **30**, 1669.
Fenton (1898). *Proc. Chem. Soc.* 119.
Gallagher (1923). *Biochem. J.* **17**, 515.
Küchlin and Boëseken (1929). *Proc. K. Akad. Wetensch. Amsterdam*, **32**, 1218.
Manchot (1902). *Liebig's Ann.* **325**, 93.
Morrell and Crofts (1899). *J. Chem. Soc.* **99**, 786.
Mummery (1913). *J. Soc. Chem. Ind.* **32**, 889.
Thurlow (1925). *Biochem. J.* **19**, 175.
Warburg (1921–1928). Papers in *Biochemische Z.*
—— (1925). *Science*, **61**, 575.

CXLVI. THE OXIDATION OF THIOSULPHATE TO SULPHATE BY TISSUE SLICES *IN VITRO*.

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SCHMIEDEBERG [1867] isolated thiosulphate (as barium thiosulphate) from the normal urine of cats and some dogs; this observation was confirmed by Meissner [1868] and by Salkowski [1886, 2] who also found it in dog faeces. Salkowski, like most later workers, did not isolate the thiosulphate but simply showed that sulphur was formed when the urine was acidified and heated. Strümpell [1876] found thiosulphate in the urine of a patient suffering from typhoid and Spiegel [1901], who suggested that it was a normal oxidation product of cystine in the body, found it in the urine of a cystinuric. Since Mester [1890] had already shown that thiosulphate occurred in the urine after feeding both cystinuric and normal men on milk of sulphur and since several workers have found it in some human urines, the presence of thiosulphate in the urine cannot be looked upon as a characteristic of any disease.

Salkowski [1872; 1873] showed that taurine was not oxidised either to sulphate or to thiosulphate in the dog, the man or the rabbit when injected, but that it was oxidised when fed to the rabbit, and he found considerable quantities of both oxidation products in the urine. He suggested that the sulphate arose secondarily by the oxidation of the thiosulphate, for Trachtenberg [1861] had already demonstrated that the latter was readily oxidised when fed to man. Salkowski later [1876] found that isethionic acid could be oxidised to sulphate by rabbits whether fed or injected but that it only gave rise to thiosulphate when fed. These observations were extended by Heffter [1886] and Rothera [1904] who pointed out the importance of the intestinal flora in the production of thiosulphate. Heffter found that putrid meat led to a greater excretion of thiosulphate when fed to dogs than fresh meat; he also showed that diets rich in carbohydrate increased thiosulphate excretion and suggested that this was due to the increased fermentation in the gut. Some of Heffter's conclusions were harshly criticised by Salkowski [1886, 1] but his main conclusions have been generally accepted.

Inspired by Mörner's demonstration that cystine occurred in proteins Blum [1903] fed cystine to dogs and Wohlgemuth [1904, 1] fed it to rabbits; both detected thiosulphate in the urine, although Goldmann, using smaller doses, had earlier [1885] failed to do so. Blum however found great individual variations between his dogs, as might be expected if the action were due to bacteria in the gut. He also showed that no thiosulphate was excreted when the cystine was given intravenously or subcutaneously. Salkowski, returning after a long interval to his old studies, found [1914, 1] that rabbits excreted more thiosulphate in their urine when fed on cabbage than when fed on other diets; he later [1914, 2; 1917] showed that the substance concerned could be extracted from cabbage by boiling water but that if the cabbage were boiled with acid

before feeding no thiosulphate was found in the urine. He demonstrated, however, that the substance was not thiosulphate itself and that it was probably not inorganic.

It would seem therefore to be a reasonable assumption that thiosulphate is either made by bacteria in the gut or else that it is made by the body from some substance that is made in the gut.

Wohlgemuth [1904, 2] found that cystine gave rise to thiosulphate when incubated with putrid meat infusion and from this argued that it was formed during intestinal putrefaction. Neuberg and Rubin [1914] similarly found that mixed putrefactive bacteria could make thiosulphate from taurine and chondroitinsulphuric acid. A mechanism is therefore available whereby organic sulphur compounds can be converted into thiosulphate but there is little reason to think that it is the only mechanism. Ever since its discovery it has been known that hydrogen sulphide is a product of protein putrefaction. Rothera [1904] found that it was made by *Bact. coli* from cystine while Almy and James [1926] and Tarr [1933, 1] have followed its production by *Proteus vulgaris*. Tarr [1933, 2; 1934] demonstrated that other bacteria, notably *Serratia marcescens* and *C. sporogenese*, had the same power and that sulphur compounds other than cystine could be broken down. The early literature of this subject is reviewed by Wilson [1923].

Hydrogen sulphide has frequently been detected in intestinal gases and this fact, taken in conjunction with McIver, Redfield and Benedict's [1926] amplification of Teschendorf's [1924] demonstration that it is very readily absorbed from body cavities, goes to show that considerable amounts are actually formed in the intestine. This process has not been studied in detail, but Deganello [1931] has shown that there is much more hydrogen sulphide in the intestine and faeces of a rabbit that is fed on cabbage than in that of one fed on other diets. This, on the theory put forward later in this paper, is in good agreement with Salkowski's [1914, 1, 2] observations. Sulphates, and so presumably other sulphur compounds, were shown by Zörkendörfer [1931] and Kochmann [1920] to be reduced when incubated with faeces, while Wilson [1923] has shown that *Bact. typhosum* and other bacteria can reduce sulphites in the same way. Many other bacteria are known which reduce inorganic sulphur compounds to hydrogen sulphide but they have been derived from sources other than the intestinal tract. Finally Lorant and Reimann [1930], who estimated the hydrogen sulphide content of many samples of faeces, have claimed that it is possible to increase the amount of faecal sulphide-sulphur by feeding iron salts and thus, by converting some of the hydrogen sulphide into an insoluble form, minimising the absorption of it. The literature that has been cited so far shows, therefore, not only that thiosulphate may be formed in the gut but that hydrogen sulphide certainly is formed in fairly large quantities and that it is absorbed.

The early literature of the effect of administration of hydrogen sulphide to animals and men has been ably summarised by Mitchell and Davenport [1924]. The recent work that has been done on the pharmacological action of the gas is not relevant from our present point of view. Flint [1887] noticed that animals could withstand a certain critical concentration of the gas without apparent ill effect and suggested that they could oxidise it to some extent. This was confirmed and extended by Wilson [1894] who found that whereas 0·1 % of hydrogen sulphide in the atmosphere would kill a rabbit in half an hour, 0·025 % had no perceptible effect; Haggard *et al.* [1922], using anaesthetised dogs, came to similar conclusions. Haggard [1921], working *in vitro* with dog's blood, found that oxygenated blood destroyed relatively large amounts of hydrogen sulphide;

he did not determine what the end-product was but concluded that it was an acid since the CO_2-binding capacity of the blood was reduced. Becher [1927] stated that all body fluids could oxidise hydrogen sulphide to thiosulphate but he gave no experimental details, nor any data as to the rate at which this oxidation proceeded; he found that boiled blood and deproteinised blood were inactive. While Becher's conclusions are very plausible the absence of experimental details makes it difficult to assess their value. Sulphhaemoglobin, which most recent workers have found to be an artefact that does not occur *in vivo*, has been thoroughly studied by Keilin [1933]. The substance previously known as sulphhaemoglobin he looks upon as a non-dissociable complex made from oxidised haemoglobin and hydrogen sulphide or an oxidation product thereof; a simple addition compound is however made by methaemoglobin with hydrogen sulphide. It is unlikely that haemoglobin itself plays any part in the oxidation of hydrogen sulphide by blood. Denis and Reed [1927] found that sodium sulphide, when injected into an anaesthetised dog in lethal or nearly lethal doses, led to an increase in the inorganic sulphate of the serum. This increase, as might be expected, was larger when the kidneys were ligatured but was always small.

The oxidation of thiosulphate to sulphate when fed to normal men was studied by Trachtenberg [1861] and Nyiri [1923, 1]; they found that a 10 g. dose could be almost completely oxidised and that only about 5 % was excreted unchanged. Nyiri [1923, 2] used the excretion of unchanged thiosulphate after its intravenous injection as an index of kidney function, for he concluded that whereas normally 30–40 % was excreted unchanged, damaged kidneys excreted 0–23 %; he estimated the thiosulphate iodimetrically after treating the urine with animal charcoal. Holbøll [1925] criticised this method and pointed out that sometimes little or no thiosulphate was excreted by people with perfectly normal kidneys. Bacterial contamination of the urine was one cause of this but a more interesting one was increased basal metabolic rate. Holbøll gives figures for the excretion by patients with Graves' disease and other metabolic disturbances or febrile conditions. Höppener [1864] had previously found that less thiosulphate was excreted in the urine when a dose was fed during periods of fever or exercise than during periods of rest. The interpretation of the results obtained by the injection of thiosulphate is therefore difficult and the fact that, as will be shown in this paper, the oxidation can take place in both liver and kidney complicates the position still further.

A study of the processes involved in the oxidation of thiosulphate in the body is of importance, not because there is any reason to suppose that thiosulphate is a normal intermediate in the breakdown of the sulphur-containing constituents of food, such as methionine and cystine, in the body, but rather because it is an instance of the body's capacity to protect itself from poisoning by the products of intestinal putrefaction and because it is the first process of this sort that has been studied *in vitro*. The extent to which sulphur compounds are broken down to thiosulphate or hydrogen sulphide in the gut is not as yet known, but the fact that, in spite of its ready oxidisability, thiosulphate is frequently found in the urine makes it probable that this process accounts for a considerable fraction of the sulphur metabolism of the animal. This type of metabolism should be especially prominent with a very luxuriant intestinal flora, and it is significant that the rabbit has been found to excrete extra sulphate in its urine after the feeding of a much larger group of sulphur compounds than is effective in this way with the dog [Sherwin *et al.*, 1927; Pirie, 1932].

Experimental.

The technique used in the series of experiments already published [Pirie, 1934] has been altered in several respects in this series of experiments. It is unnecessary, when studying the oxidation of thiosulphate, to cut the tissue slices as thin as Warburg recommends, for there is no detectable difference in the amount of sulphate formed per mg. dry weight of tissue when the slices are cut as thinly as possible and when relatively coarse (0·5–0·7 mm.) slices are used. It is unnecessary also to use the complicated Ringer solution used by Krebs [1932], for this oxidation takes place as readily in a mixture of isotonic sodium chloride solution and isotonic sodium bicarbonate of the correct p_H. The p_H generally used was 7·6 but, as will be shown later, this need not be adhered to closely.

The method used for the estimation of sulphate is still somewhat unsatisfactory. In my previous paper the method of Cuthbertson and Tompsett [1931] was modified, and the precipitate of benzidine sulphate was washed on a filter instead of on the centrifuge. This change was made because of the difficulty experienced in making a small pellet of benzidine sulphate adhere to the end of a centrifuge-tube. This difficulty has now been overcome by the use of a faster centrifuge (3500 R.P.M.) and by washing the precipitate with 80 % acetone instead of with absolute acetone. In some recent experiments a relatively large amount of cystine and of certain tissue extractives has been precipitated along with the benzidine sulphate; this precipitate is very readily washed free from absorbed benzidine on the centrifuge but less easily on a filter. In the method of estimation used at present the technique of precipitating the benzidine sulphate and of estimating the benzidine colorimetrically is that described before, while the method of washing is that of Cuthbertson and Tompsett.

Benzidine forms a thiosulphate that is similar in many respects to its sulphate [Wahl, 1902]. This precipitates when there is more than 0·7 mg. of sodium thiosulphate in the 2 ml. sample on which sulphate is estimated. The thiosulphate is however much more soluble in 80 % acetone than benzidine sulphate and so is dissolved during the washing. The concentration of sodium thiosulphate may therefore be raised to 1 or 1·5 mg. in the 2 ml. sample without affecting the sulphate estimations. It is well known that thiosulphates are not stable in acid solution but break down to give free sulphur and a not very clearly defined group of sulphur acids; fortunately these breakdown products do not appear to precipitate with benzidine in dilute solution. Sulphate can therefore be estimated in the usual way in a solution from which the protein has been removed with trichloroacetic acid. The sulphite that is formed during the acid decomposition of thiosulphate is fairly rapidly oxidised to sulphate. The sulphate values will therefore be too high unless the estimations are started not more than half an hour after the removal of the protein. It would appear that there is no oxidation after the addition of the acetone and benzidine, for the same sulphate figures are obtained whether the estimations are left at this stage for 2 or 20 hours.

Oxidation of thiosulphate by rat tissues.

Some experiments on the oxidation of sodium thiosulphate by slices of rat tissue are collected in Table I *a*. Only liver, kidney and chorion have been found to bring about the oxidation rapidly under the conditions used, and the first is the most active. Brain, testis, spleen, blood, muscle (diaphragm) and placenta are inactive or only very feebly active. The livers used in Exps. 95 and 12

(Table I *a*) were from foetal rats about 2 and 5 days from term respectively. Clearly, foetal liver is active but less so than adult liver. It is obvious from Table I *a* that liver varies markedly in activity, and so far it has not proved possible to correlate this variation with differences in age, sex *etc.* in the rats.

The effect of inhibitors on this oxidation has not yet been studied in any detail, but the effect of a number of physical agents is recorded in the hope that at a later stage it may lead to an understanding of the mechanism of this oxidation.

Heating for 10 minutes to 60° inactivates the slices completely, and slices that have been kept at 0° for more than 2 days are inactive. These facts, taken in conjunction with the inactivity of organs other than liver and kidney, tend to confirm the theory that we have here to do with a real enzyme action and not simply with a catalytic oxidation. The enzyme system responsible for this oxidation is more stable than that concerned in the oxidation of cysteine to sulphate. Liver, for example, whether whole or sliced, will keep its activity almost unimpaired for 8–10 hours if kept cold, and the same slices which have already been used in one oxidation experiment lasting 4 hours may be used over again and show only a partial falling off in their activity. The liver used in Exp. 85 (Table I *a*) is a case in point for it had already been shaken for 4 hours at 37° with thiosulphate before Exp. 85 was put up.

A further instance of the relative stability of this system is given by its behaviour towards hypotonic solutions. Table I *b* illustrates this, for slices of the same organ are put up at the same p_H and for the same time but with the sodium chloride solution replaced by distilled water in some cases so that in these cases the tissue is suspended in a fluid with only one-sixth the normal osmotic pressure. In Exp. 75 both the oxidations were carried out under isotonic conditions but the slices used in the one marked "hypotonic" had been left with occasional shaking in distilled water for 3 hours before use. If a large volume of cold water is used and it is changed frequently the inactivation is greater. For example, the 60 mg. dry weight of slices used in Exp. 19 were washed for an hour with 250 ml. of ice-cold distilled water; after a second and a third washing with fresh cold water the activity was found to be only one-third of that of the untreated slices. Exposure to hypertonic solutions, *e.g.* 9·0 % sodium chloride solution, causes at 0° a rapid diminution in the activity of liver slices. The dry weights of tissue slices that have been shaken isotonically and hypotonically are not strictly comparable, for in the former case the wet weight is about 8·3 times the dry whereas in the latter it is about 10 times but variable. This difference in the amount of material soluble under the two conditions also shows up as a marked increase in the amount of material precipitable by trichloroacetic acid in the hypotonic fluid. Measurements of the dry weight of liver are also more or less invalid in all experiments done at a greater p_H than 8 or for periods longer than 2 hours. In spite of its apparent stability all attempts to make a preparation of this enzyme have so far been unsuccessful. Fine mincing of the liver causes a marked fall in the activity, as does freezing, while thorough grinding with sand leads to an almost complete loss of activity. It seems, therefore, that any damage to the cell structure leads to partial inactivation, and this is borne out by the fact that treatment with toluene or chloroform reduces markedly the capacity of liver to perform this oxidation. Mere subjection of the slices to high pressure on the other hand has little effect, and a sample that had been exposed to a pressure of 40 atmospheres of nitrogen for 90 minutes had Q 4·4 whereas the control Q was 5·9.

The kinetics of the oxidation of thiosulphate by rat-liver have been studied in some detail. Exps. 62 and 73 in Table I *a* show that, as might be expected,

the amount of sulphate formed is proportional to the time of action and to the amount of liver present. This regularity justifies the introduction of the symbol Q to indicate thousandths of a mg. of sulphur appearing in the form of sulphate per hour per mg. dry weight of tissue. Since Q varies with the concentration of substrate as well as with the nature of the substrate it is necessary to introduce a suffix to represent the concentration (expressed in thousandths of a mg. of sulphur per ml.) and nature of the substrate. The symbol for the conditions most commonly used in this paper, *i.e.* 3·5 mg. of hydrated sodium thiosulphate in 8 ml. of solution, is therefore $Q_{112\ \text{thio}}$.

Table I *a*.

Exp. No.	Wt. of thiosulphate in 8 ml. (mg.)	Dry wt. of tissue (mg.)	Time of action min.	Wt. of sulphur oxidised to sulphate (γ)	Q
62	3·7	101 liver	30	140	2·7
	3·7	107	60	275	2·6
	3·7	83	120	420	2·5
73	4·1	10	120	55	2·7
	4·1	26	120	100	1·9
	4·1	53	120	225	2·2
85	3·5	100 (old)	120	265	1·3
90	3·5	33	64	130	3·7
95	3·5	23 } foetal	120	73	1·6
12	3·5	35 } liver	120	72	1·0
97	3·5	52	60	185	3·5
	3·5	73	60	225	3·1
63	3·7	82 kidney	120	245	1·5
85	3·5	40 kidney	120	200	2·5
12	3·5	20 chorion	113	78	2·1
15	3·5	17 chorion	90	64	2·5

Table I *b*.

62	3·7	Isotonic 83 liver	120	420	2·5
		Hypotonic 63	,,	400	3·2
75	3·3	Isotonic 45	120	175	2·0
		Hypotonic 76	,,	200	1·3
84	3·5	Isotonic 47	120	225	2·3
		Hypotonic 43	,,	166	1·9
85	3·5	Isotonic 40 kidney	120	200	2·5
		Hypotonic 25	,,	105	2·1
90	3·5	Isotonic 33 liver	60	130	3·7
		Hypotonic 35	,,	145	4·1
94	3·5	Isotonic 56	60	180	3·2
		Hypotonic 56	,,	120	2·1
19	3·5	Isotonic 45	60	270	6·0
	3·5	Hypotonic 60	,,	114	1·9

Table I *c*.

62	3·7	83 liver	120	420	2·5
	1·8	77	120	310	2·0
73	4·1	5·3	120	225	2·2
	2·0	75	120	200	1·3
	0·8	56	120	105	0·9
97	3·5	52	60	185	3·5
	2·7	43	60	148	3·4
	1·8	40	60	110	2·7
	0·9	42	60	65	1·5

The effect of changes in the concentration of thiosulphate is rather less simple, for the amount of sulphate formed is not quite doubled when the thiosulphate concentration is doubled. This is shown in Table I *c*. It is clear from this table that in any experiment in which much of the thiosulphate initially present has been oxidised the Q value will be smaller than it should be on account of the fall in substrate concentration. For this reason and also on account of the progressive inactivation of slices at 37° it is advisable to calculate Q values from short (1 hour) experiments.

At p_H values between 7·3 and 8 the rate of formation of sulphate is approximately constant but it falls off suddenly on either side of this range. On the acid side this diminution in activity is complicated by the spontaneous oxidation of the thiosulphate at a slow rate in even weakly acid solution. Two typical experiments on rat-liver are recorded in Table II in one of which only the

Table II.

p_H	Wt. of thiosulphate (mg.)	Dry wt. of rat-liver (mg.)	Time mins.	Wt. of sulphur oxidised to sulphate (γ)	Q
6·7	3·5	72	60	120	1·6 (1·8)
7·0	,,	69	,,	120	1·7 (2·6)
7·3	,,	65	,,	190	2·9 (2·9)
7·6	,,	73	,,	225	3·1 (3·0)
7·9	,,	62	,,	230	3·7 (3·1)
8·2	,,	(51)	,,	185	—
		Guinea-pig-kidney			
6·6	3·5	66	60	75	0·8
6·8	,,	61	,,	72	0·9
7·1	,,	95	,,	105	0·8
7·7	,,	79	,,	148	1·4
8·0	,,	57	,,	140	1·8
8·3	,,	(43)	,,	75	—

Q values are given. The p_H was controlled by varying the ratio of isotonic sodium bicarbonate to isotonic sodium chloride and, in the case of the more alkaline solutions, by using 97·5 % oxygen and 2·5 % carbon dioxide in place of the usual 95 % oxygen and 5 % carbon dioxide mixture.

In a few experiments the amount of thiosulphate disappearing was measured as well as the amount of sulphate formed. As can be seen in Table III the

Table III.

Wt. of thiosulphate in 8 ml. (mg.)	Dry wt. of rat-liver (mg.)	Time of action (mins.)	Sulphur in thiosulphate oxidised (γ)	Sulphur in sulphate formed (γ)	Q
3·5	71	240	520	430	1·5
,,	60	120	248	230	1·9
,,	56	60	146	180	3·2
,,	50*	60	180	170	3·4
,,	56†	60	139	120	2·1

* Slices cut very thinly. † Hypotonic.

agreement is reasonably good. The thiosulphate was estimated by removing the protein from a sample of the reaction mixture with zinc sulphate and sodium hydroxide as in the blood-sugar method of Hagedorn and Jensen. An excess of an acid solution of iodine in potassium iodide was then added and the excess

titrated with standard thiosulphate solution. Tissue extractives such as glutathione will of course interfere, but under the aerobic conditions used in these experiments such substances will be largely in the oxidised state, and in any case they are quantitatively insignificant. A more serious error is caused by the fact that only one-third or one-fourth of the thiosulphate is oxidised in most experiments and one is therefore measuring, rather inexactly, a small change in a fairly large quantity. It is clear from Table III that, allowing for the probable errors, there is no evidence for the existence, under these conditions, of any process leading to the destruction of appreciable amounts of thiosulphate except the formation of sulphate. This demonstration raises the question of the possible rôle of the best known oxidation product of thiosulphate, namely, sodium tetrathionate, in this oxidation. Sodium tetrathionate was prepared in the usual way by pouring a strong aqueous solution of sodium thiosulphate into an alcoholic solution of iodine. The precipitated tetrathionate was washed well with alcohol, taken up in water and precipitated again with alcohol; it had the theoretical sulphur content. As Table IV *a* shows, sodium tetrathionate is oxidised to sulphate by rat-liver and kidney but at a very much slower rate, considering the amount of sulphur that is present in 1 ml. of solution, than sodium thiosulphate, *i.e.* $Q_{143\ \text{tetra}}$ is 1·4–3·2 whereas $Q_{112\ \text{thio}}$ is 2·5–5·9. It is therefore unlikely that tetrathionate is an intermediate in the oxidation of thiosulphate to sulphate. It is possible that oxidation to sulphate is preceded by a reduction of the tetrathionate to thiosulphate. I showed in the earlier paper that such a preliminary reduction took place in the case of the oxidation of cystine but so far I have been unable to demonstrate it in this case. It is unlikely that such a reduction takes place, for the ratio $Q_{\text{thio}}/Q_{\text{tetra}}$ appears to be constant irrespective of the treatment that the slices of liver have undergone. In the case of cystine, on the other hand, it was found that the capacity of liver slices to reduce cystine to cysteine was strictly limited and that this limited the production of sulphate. The analogy is of course not wholly satisfactory, since thiosulphate, unlike cysteine, cannot undergo aerobic re-oxidation, and so it might be expected that it would be easier for the liver to maintain the concentration of the reduced substance. When incubated anaerobically with liver slices, sodium tetrathionate does not give rise to any appreciable amount of thiosulphate. Some further experiments illustrating this type of oxidation are given in Table IV *b*. All the experiments lasted for an hour and were carried out in 97·5 % O_2, 2·5 % CO_2, but the slices used in Exps. *c* and *d* are those that had already been used in *a*, while those used in *e* and *f* had already been used in *b*. Clearly the $Q_{\text{thio}}/Q_{\text{tetra}}$ ratio is approximately constant, and previous exposure to tetrathionate leads to no greater loss of activity than exposure to thiosulphate. The other thionic acids have not been tried, and sodium sulphite is so rapidly autoxidised in the absence of tissue, and still more in the presence of boiled tissue, that a study of its enzymic oxidation is not possible by this technique.

The falling off in the activity of a tissue during the second hour of shaking is especially marked in the case of livers which are initially very active. Such a fall is clearly seen in Tables III and IV *b* and is analysed more fully in Table IV *c*. There are three possible explanations of this phenomenon; firstly it may simply be the normal ageing process accelerated by the higher temperature at which the liver has been kept; secondly it may be due to the using up of a metabolite during the course of the oxidation of thiosulphate; and thirdly it may be due to the loss by diffusion of a soluble substance, either a metabolite or a co-enzyme, essential for sulphate formation. The results recorded in Table IV *c* are strongly

Table IV *a*.

Exp. No.	Wt. of tetrathionate (mg.)	Dry wt. of tissue (mg.)	Time of action (mins.)	Wt. of sulphur oxidised to sulphate (γ)	Q
84	2·4	56 liver	120	165	1·4
	2·4	45 liver heated to 70°	,,	5	—
85	2·4	42 kidney	,,	95	1·1
	2·4	28 kidney heated	,,	6	—
	2·4	No tissue	,,	0	—
90	2·7	42 liver	64	92	2·0
	5·4	40	,,	125	2·9
	1·3	41	,,	70	1·6
	2·7 (hypotonic)	43	,,	108	2·3
3	2·4	91 liver	60	160	1·8
4	2·4	37 liver	60	45	1·2

Table IV *b*.

7 *a*	3·5 thio.	53 liver	60	315	5·9
b	2·4 tetra.	57 liver	,,	185	3·2
c	3·5 thio.	24 liver	,,	73	3·0
d	2·4 tetra.	29 liver	,,	45	1·5
e	3·5 thio.	22 liver	,,	75	3·2
f	2·4 tetra.	35 liver	,,	55	1·6

Table IV *c*.

11 *a*	3·5 thio.	43 liver	60	240	5·6
b	3·5	43	120	400	4·7
c	3·5	43	60	185	4·3
d	3·5	37	,,	160	4·3
e	3·5	29	,,	115	4·0
f	3·5	45	75	120	2·1
g	3·5	28	,,	55	1·6
12 *a*	3·5	59	60	95	1·6
b	3·5	52	,,	102	2·0

in favour of the first theory. Exps. *a* and *b* were carried out in the usual way for periods of 1 and 2 hours respectively. Exp. *c* is a 1 hour experiment on the slices that had already been used in Exp. *a*. This gives $Q=4{\cdot}3$ instead of the initial 5·6. The Q values found in Exps. *d* and *e* should be compared with this value for in each case the slices underwent a preliminary shaking for one hour in thiosulphate-free bicarbonate-saline. After this, thiosulphate was added to the one (*d*) while the slices were removed from the other, rinsed and added to fresh solution containing thiosulphate. Clearly the values of Q in Exps. *c*, *d* and *e* are very similar; this shows that the presence of thiosulphate leads to no increase in the rate of disappearance of essential metabolites, for if it did Q in Exp. *c* would be smaller than in Exps. *d* or *e*. Similarly there is little loss by diffusion for if there were *e* should be smaller than *d*. Exps. *f* and *g* were carried out in the same way but with 2 hours' preliminary shaking at 37°. In Exp. *f* the oxidation was carried out in the fluid in which the slices had previously been shaken for 90 minutes, while in *g* fresh fluid was used. The difference, though definite, is small enough to indicate that this is not an important cause of loss of activity. If a gas mixture consisting of 95 % N_2 and 5 % CO_2 is used for the preliminary shaking, it is found that the slices are completely inactivated in an hour whether thiosulphate is present or not. Anaerobic incubation for half an hour causes nearly complete inactivation. Other oxidising systems are

already known which are stable only in the presence of oxygen, but this one is specially interesting since, as will be shown later, the oxidation of thiosulphate does not lead to any increase in the oxygen consumption of the slices. On account of this inactivation there is no anaerobic formation of sulphate. The presence of $M/4000$ methylene blue and $M/200$ nitrate leads neither to the production of sulphate, although the methylene blue is kept in the oxidised state by the nitrate-reducing system in the liver, nor to an increase in the stability of the enzyme system. Methylene blue and nitrate, whether singly or together, have also no effect on the aerobic sulphate production.

Manometric experiments with rat-liver.

In collaboration with Dr H. A. Krebs of this laboratory some experiments on the influence of thiosulphate on the oxygen uptake of liver have been carried out. These have led to the unexpected result that, under conditions where oxygen is appearing in the form of sulphate at a rate corresponding to one-third of the total respiration, there is no apparent increase in the oxygen consumption. Table V gives the results of one such experiment. The fluid used was Ringer

Table V.

Wt. of tissue (mg.)	Wt. of thiosulphate in 3 ml. Ringer (mg.)	Wt. of sulphur oxidised to SO_4 per hour (γ)	Q_{thio}	Oxygen equivalent of this sulphur reduced to 40 mins. (μl.)	Oxygen consumption in 40 mins. (observed) (μl.)	Q_{O_2}
7·9	1·3	27	3·4	15·7	44·7	8·5
9·7	1·3	31	3·2	18·0	56	8·7
16·1	None	—	—	—	95	8·85
7·9	None	—	—	—	45·5	8·6

solution made up according to Krebs [1932] but with magnesium sulphate replaced by magnesium chloride and with only 2 ml. of 1·2 % sodium bicarbonate per 100. Such a solution maintains a suitable p_H when used in conjunction with pure oxygen and with a side-bulb to absorb CO_2. Sulphate estimations were done on the reaction fluid in the usual way and the oxygen equivalent is calculated on the basis of 5 oxygen atoms to 2 atoms of sulphur as required by theory. This value is multiplied by 2/3 since the oxygen absorbtion was only followed for 40 minutes while the tissue was in contact with thiosulphate at 37° for an hour. The constancy in the values of Q_{O_2} in the four experiments recorded shows that no extra oxygen can have been taken up during the oxidation of the thiosulphate.

Oxidation by the tissues of other animals.

A few experiments have been made with other animals. It is unnecessary to give the results in detail and the Q values only are recorded.

	Q
Ox-kidney (2–3 hours after slaughter)	1·3
Rabbit-liver...	1·3
Rabbit-kidney	0·65
Guinea-pig-kidney	1·1–2·7
Pig-liver (2–3 hours after slaughter)	1·0
Dog-kidney (after chloroform)	0·6
Dog-liver (after chloroform)	0·8

All these experiments were done at p_H 7·6 and with 112γ sulphur present in the form of thiosulphate per ml. The effect of changes in p_H on the activity of

guinea-pig-kidney is recorded in Table II whence it is clear that the enzyme system found there is, from this point of view, similar to that found in rat-liver. The other tissues have not been studied further.

Oxidation by goose-kidney.

Goose-kidney resembles rat-liver closely in its ability to oxidise thiosulphate to sulphate. The results of a few experiments are given in Table VI; these were

Table VI.

Exp. No.	Wt. of thiosulphate (mg.)	Wt. of goose-kidney (mg.)	Time of action (mins.)	Wt. of sulphur oxidised to SO_4 (γ)	Q
78	4·0	90	120	650	3·6
79	4·0	55	120	360	3·3
	4·0	25	120	160	3·2
	2·4	68	120	280	2·1
	1·2	70	120	180	1·3
88 p_H 6·7	3·5	72	120	385	2·8
p_H 7·3	3·5	46	120	300	3·3
p_H 7·7	3·5	65	120	410	3·2
p_H 8·0	3·5	54	120	380	3·5

carried out in the usual way at p_H 7·6 but using a mixture of 1·2 % sodium bicarbonate and 1·1 % sodium chloride as the reaction fluid. As with rat organs no sulphate is made by slices that have been heated to 60° nor if the slices are shaken with thiosulphate anaerobically. Freezing and grinding cause a very marked diminution in the activity while the effect of treatment with distilled water is definite though less marked.

Goose-kidney also resembles rat-liver and kidney in its ability to oxidise cysteine and methionine to sulphate; this oxidation will be described in a later paper.

Discussion.

Although the oxidation of thiosulphate by bacteria was first observed many years ago the mechanism of the process is still obscure. Several species of bacteria are known which have this power. Lieske [1912] and Guittonneau [1925, 1, 2] have isolated such organisms from pond mud and soil but they did not study the oxidation in any detail. Nathanssohn [1902] and Trautwein [1921; 1924] have studied similar bacteria and find that the oxidation proceeds through tetrathionate and dithionate. Beijerinck [1904], on the other hand, working with *Thiobacillus thioparus*, found that tetrathionate was less readily oxidised than thiosulphate. The behaviour of this organism is therefore analogous to that of liver. A comparative study of the properties and stability of the enzyme systems derived from these bacteria and from liver would be very interesting.

Thiosulphate can scarcely be oxidised directly, for in that case 5 atoms of oxygen would have to be present simultaneously for the oxidation of one molecule of thiosulphate; furthermore, if the oxidation were direct it would be hard to see why it should cause no increase in the oxygen consumption of liver slices. The irreversible inactivation of liver slices on exposure to nitrogen is especially interesting in this connection. It is known that the respiration of cells is, in general, irreversibly inhibited by anaerobic incubation and Warburg *et al.* [1926] have found that even tumour tissue will not respire after 4 hours without oxygen or glucose. These facts suggest that the oxidation of thiosulphate

is a phenomenon which parallels closely the fundamental oxidation processes of the cell, but the connection is incomplete since some organs, with relatively high respiration rates, cannot bring about this oxidation. All attempts to reactivate liver that has been inactivated in this way have so far been unsuccessful but further work on this problem is in progress.

Summary.

Rat-liver, kidney and chorion, and goose-kidney, can oxidise sodium thiosulphate to sulphate.

This oxidation is accompanied by no perceptible increase in the oxygen consumption of the tissue slices, but it will not proceed anaerobically. The slices are irreversibly inactivated by anaerobic incubation.

Sodium tetrathionate is also oxidised, but reasons are given for thinking that it is not an intermediate.

The reason for the loss of activity by the slices on keeping and the effects of some physical agents on the enzyme system have been investigated.

The liver and kidney of some other animals have been tested and found to be active but relatively weak.

REFERENCES.

Almy and James (1926). *J. Bact.* **12**, 319.
Becher (1927). *Münch. med. Woch.* **74**, 1950.
Beijerinck (1904). *Cent. Bakt. Par.* II, **11**, 593.
Blum (1903). *Hofmeister's Beitr.* **5**, 1.
Cuthbertson and Tompsett (1931). *Biochem. J.* **25**, 1237.
Deganello (1931). *Arch. Farm. Sperim.* **52**, 181.
Denis and Reed (1927). *J. Biol. Chem.* **72**, 385.
Flint (1887). *Med. News*, Philadelphia, 670.
Goldmann (1885). *Z. physiol. Chem.* **9**, 260.
Guittonneau (1925, 1). *Compt. Rend. Acad. Sci.* **180**, 1142.
—— (1925, 2). *Compt. Rend. Acad. Sci.* **181**, 261.
Haggard (1921). *J. Biol. Chem.* **49**, 519.
—— Henderson and Charlton (1922). *Amer. J. Physiol.* **61**, 289.
Heffter (1886). *Pflüger's Arch.* **38**, 476.
Holboll (1925). *Klin. Woch.* **4**, 1636.
Höppener (1864). *Schmidt's Jahrb.* **123**, 147.
Keilin (1933). *Proc. Roy. Soc. Lond.* B **113**, 393.
Kochmann (1920). *Biochem. Z.* **112**, 255.
Krebs (1932). *Z. physiol. Chem.* **210**, 33.
Lieske (1912). *Ber. deutsch. bot. Ges.* **30**, 12.
Lorant and Reimann (1930). *Biochem. Z.* **228**, 300.
McIver, Redfield and Benedict (1926). *Amer. J. Physiol.* **76**, 92.
Meissner (1868). *Z. rat. Med.* **31**, 322.
Mester (1890). *Z. physiol. Chem.* **14**, 109.
Mitchell and Davenport (1924). *U.S. Pub. Health Rep.* **39**, 1.
Nathanssohn (1902). *Mitt. Zoo. Stat. Neapel*, **15**, 665.
Neuberg and Rubin (1914). *Biochem. Z.* **67**, 82.
Nyiri (1923, 1). *Biochem. Z.* **141**, 160.
—— (1923, 2). *Klin. Woch.* **2**, 204.
Pirie (1932). *Biochem. J.* **26**, 2041.
—— (1934). *Biochem. J.* **28**, 305.
Rothera (1904). *J. Physiol.* **32**, 175.

Salkowski (1872). *Ber. deutsch. chem. Ges.* **5**, 637.
—— (1873). *Virchow's Arch.* **58**, 460.
—— (1876). *Virchow's Arch.* **66**, 315.
—— (1886, 1). *Pflüger's Arch.* **39**, 209.
—— (1886, 2). *Z. physiol. Chem.* **10**, 106.
—— (1914, 1). *Z. physiol. Chem.* **89**, 485.
—— (1914, 2). *Z. physiol. Chem.* **92**, 89.
—— (1917). *Biochem. Z.* **79**, 68.
Schmiedeberg (1867). *Arch. Heilk.* **8**, 422.
Sherwin, Shiple and Rose (1927). *J. Biol. Chem.* **73**, 607.
Spiegel (1901). *Virchow's Arch.* **166**, 364.
Strümpell (1876). *Arch. Heilk.* **17**, 390.
Tarr (1933, 1). *Biochem. J.* **27**, 759.
—— (1933, 2). *Biochem. J.* **27**, 1869.
—— (1934). *Biochem. J.* **28**, 192.
Teschendorf (1924). *Arch. exp. Path. Pharm.* **104**, 352.
Trachtenberg (1861). Dissertation, Dorpat.
Trautwein (1921). *Cent. Bakt. Par.* II, **53**, 513.
—— (1924). *Cent. Bakt. Par.* II, **61**, 1.
Wahl (1902). *Bull. Soc. Chim.* (3), **27**, 1221.
Warburg, Wind and Negelein (1926). *Klin. Woch.* **5**, 829.
Wilson (1894). *Chem. News*, **69**, 159.
—— (1923). *J. Hyg.* **21**, 392.
Wohlgemuth (1904, 1). *Z. physiol. Chem.* **40**, 81.
—— (1904, 2). *Z. physiol. Chem.* **43**, 469.
Zörkendörfer (1931). *Arch. exp. Path. Pharm.* **161**, 437.

CXLVII. THE RELATION OF THE COLOUR AND VITAMIN A CONTENT OF BUTTER TO THE NATURE OF THE RATION FED.

I. INFLUENCE OF THE RATION ON THE YELLOW COLOUR OF THE BUTTER.

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II. THE CAROTENOID AND VITAMIN A CONTENTS OF THE BUTTER.

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I. INFLUENCE OF THE RATION ON THE YELLOW COLOUR OF THE BUTTER.

Under the normal system of feeding dairy cows in winter in this country a most noticeable feature is the pale colour of the milk, a fact greatly emphasised in the butter made from it. The intensity of the yellow colour of the milk fat is positively correlated [Watson *et al.*, 1933] with the carotene content of the butter and also with its vitamin A content. The inclusion in the ration of some foodstuff capable of increasing the yellow colour of the milk fat is therefore of considerable importance. The oil cakes and cereals in common use are incapable of bringing about this result, for which the use of some foodstuff rich in carotene is needed. Carrots themselves may be used, but there are difficulties in the way of their adoption in general practice. In one case examined the milk of the herd produced a butter with a total yellow colour of 3·2 Lovibond units[1] which was raised to 7·2 units by feeding about 10 lb. of carrots per head daily.

In an experiment carried out in the winter of 1931–32 [Watson *et al.*, 1933] and also reported in this *Journal* [Gillam *et al.*, 1933], it was shown that artificially dried grass was a useful foodstuff in this regard when it formed 75 % of the total concentrated foodstuffs fed. This work has been extended in 1932–33 to test the value of different methods of conservation of pasture grass and their effect, in the ration, on the colour of butter.

[1] The yellow colour is stated in all cases as the Lovibond yellow units corresponding to a 1 cm. thickness of the fat. For the measurement it is diluted with three times its volume of light petroleum, the actual reading being multiplied by four.

In the first experiment four groups of Shorthorn cows were used. All groups received a basal ration of hay and roots. Group D1 acted as control and received a normal winter ration of concentrated foodstuffs, balanced for milk production, at the rate of $3\frac{1}{2}$ lb. per gallon of milk produced. Groups E1, E2 and E3 received 10 %, 25 % and 50 % respectively of their concentrate ration in the form of artificially dried grass, the mixture being so adjusted that the same amounts of nutrients (starch equivalent and protein equivalent) were fed per gallon of milk to all four groups. The values obtained for the total yellow colour of the butter are given in Table I, and are shown in Fig. 1.

Table I. *Total yellow colour of the butter from groups of Shorthorn cows on a normal winter ration, and on rations containing varying amounts of artificially dried grass.*

Stated as Lovibond units.

Week ending	D 1 Control	E 1 (10 % dried grass)	E 2 (25 % dried grass)	E 3 (50 % dried grass)
17. xi. 32*	6·8	8·8	8·0	7·2
15. xii. 32	7·2	8·4	9·2	12·8
29. xii. 32	6·8	8·4	8·4	12·0
23. i. 33	6·0	7·6	7·6	10·0
13. ii. 33	4·4	6·0	6·0	9·2
2. iii. 33	4·8	6·4	5·2	8·4
27. iii. 33	5·2	6·0	5·2	9·6
Averages	5·7	7·1	6·9	10·3

* All four groups on control ration.

The total yellow colour of the butter has not been markedly affected by the inclusion of 10 and 25 % of artificially dried grass in the concentrate ration, but the group receiving 50 % of its concentrates in the form of this material shows a much greater improvement in this respect.

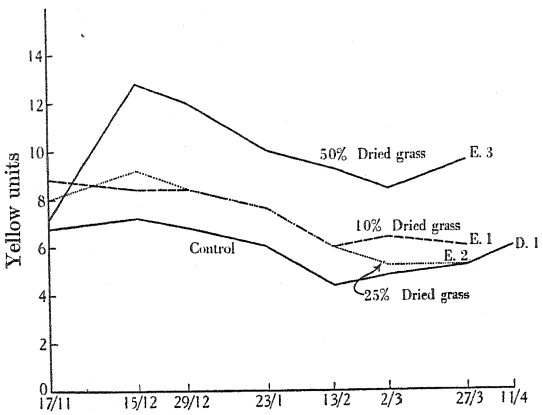

Fig. 1.

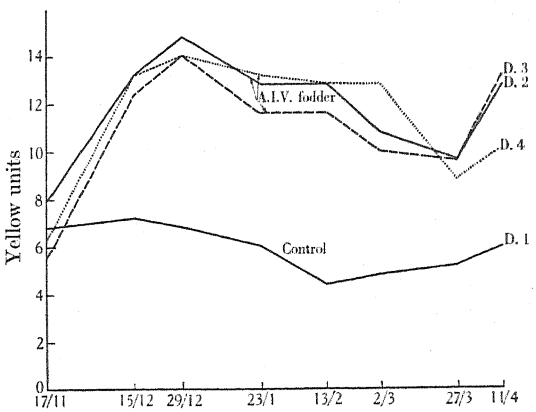

Fig. 2.

In the next experiment four pairs of cows were selected from the Shorthorn herd. One of these (group D1 *supra*) received an ordinary winter ration of hay, roots and concentrates, group D2 had a part of these replaced by 40 lb. of A.I.V. fodder, D3 by 70 lb. of A.I.V. fodder, whilst D4 received no purchased

foodstuffs but only A.I.V. fodder, hay and artificially dried grass, all four rations being of equivalent nutritive value (starch equivalent and protein equivalent).

A.I.V. fodder is produced by a modification of the ensilage process in which a forage crop—grass in this case—is treated with a dilute solution of mineral acids in such amounts that an acidity of p_H 3–4 is developed in the mass, which is stored in a suitable container to which the free access of air is prevented [Virtanen, 1933]. The temperature of the mass does not rise appreciably, and the carotene content of the green crop is retained in a large measure, whereas in the case of the warm fermentation process of ensilage, in which the temperature rises to the neighbourhood of 50°, most of the pigment is destroyed.

The test continued throughout the winter and the results are given in Table II in terms of the total yellow colours of the butters made from the milk.

Table II. *Total yellow colour of the butter from groups of Shorthorn cows on a normal winter ration, and on rations containing varying amounts of A.I.V. fodder.*

Stated as Lovibond units.

Week ending	D 1 Control	D 2 (40 lb. A.I.V.)	D 3 (70 lb. A.I.V.)	D 4 (70 lb. A.I.V. + dried grass)
17. xi. 32*	6·8	8·0	5·6	6·4
15. xii. 32	7·2	13·2	12·4	13·2
29. xii. 32	6·8	14·8	14·0	14·0
23. i. 33	6·0	12·8	11·6	13·2
13. ii. 33	4·4	12·8	11·6	12·8
2. iii. 33	4·8	10·8	10·0	12·8
27. iii. 33	5·2	9·6	9·6	8·8
11. iv. 33	6·0	12·8	13·2	10·0
Averages	5·8	12·4	11·8	12·1

* All four groups on control ration.

These results are also shown graphically in Fig. 2. The control group D1 produced butters which were pale in colour until the cows were turned out to pasture in April, when the colour value began to rise. The feeding of 40 lb. of A.I.V. fodder greatly increased the total yellow colour of the butter, whilst the 70 lb. of A.I.V. fodder were of equal value in this regard, but no better than 40 lb. Group D4 (A.I.V. fodder, artificially dried grass and hay only) showed no further advantage over the other two experimental groups. The rise in colour in April coincided with the advent of fresh grass.

Table III. *Carotene contents of A.I.V. fodder and artificially dried grass.*

Stated as mg./100 g. of the dry weight.

Date	A.I.V. fodder	Date	Artificially dried grass
23. xi. 32	52·2	6. xii. 32	47·9
13. xii. 32	33·9	20. xii. 32	38·0
11. i. 33	44·1	28. xii. 32	35·9
24. i. 33	38·1	11. i. 33	35·2
28. ii. 33	47·2	26. i. 33	40·1
21. iii. 33	40·8	6. ii. 33	34·4
—	—	10. ii. 33	46·9
—	—	16. ii. 33	36·0
—	—	24. ii. 33	26·6
—	—	3. iii. 33	26·7
—	—	10. iii. 33	28·4
—	—	16. iii. 33	34·5
Average	42·7	Average	35·9

The comparative effects of the rations of dried grass and of A.I.V. fodder in these experiments are shown in Fig. 3, where the group receiving 50 % of its concentrate ration as dried grass (E3) is compared with that receiving 40 lb. of A.I.V. fodder (D2) and with the control group (D1) in all of which the total nutrient values (S.E. and P.E.) of the rations were equal. The A.I.V. fodder has been more effective, but throughout the trial the cows on this diet were receiving a greater amount of dry matter and of carotene daily in the form of A.I.V. fodder than was fed in the form of artificially dried grass. The A.I.V. fodder supplied on the average 10 lb. of dry matter per head daily, whereas the dried grass supplied about 6 lb.

The carotene contents of the two foodstuffs were determined at intervals and the results are given in Table III.

In another experiment four pairs of Ayrshire cows were used (F1 to F4). The control group F4 received a normal winter ration similar to that fed to group D1 of the Shorthorns. To groups F1 and F3 were fed 40 lb. per head daily of A.I.V. fodder made with different acid mixtures, whilst group F2 received 40 lb. per head daily of a fodder made by the addition of a weaker acid mixture, sufficient to raise the acidity of the mass only to p_H 4·5, together with some molasses to stimulate a lactic acid fermentation.

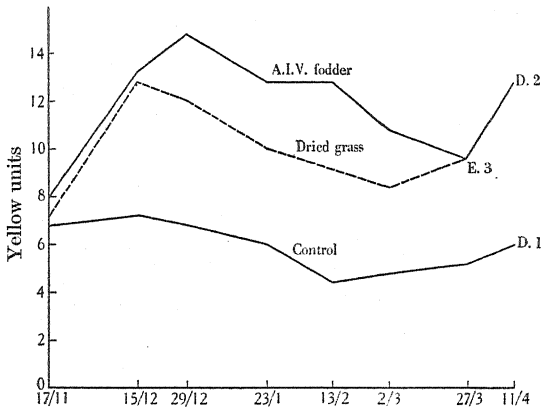

Fig. 3.

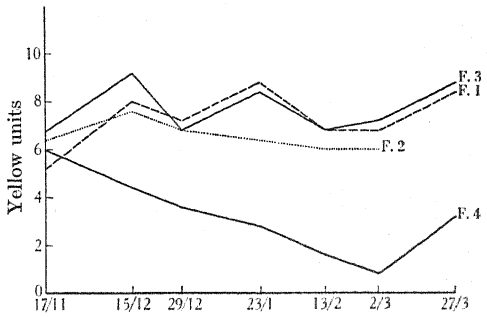

Fig. 4.

The results of this experiment are given in Table IV in terms of the total yellow colour of the butters.

Table IV. *Total yellow colour of the butter from groups of Ayrshire cows on a normal winter ration and on rations containing different types of acid-treated green fodder.*

Week ending	F 1 (A.I.V. fodder No. 1)	F 2 (Acid + molasses fodder)	F 3 (A.I.V. fodder No. 2)	F 4 Control
17. xi. 32*	5·2	6·4	6·8	6·0
15. xii. 32	8·0	7·6	9·2	4·4
29. xii. 32	7·2	6·8	6·8	3·6
23. i. 33	8·8	6·4	8·4	2·8
13. ii. 33	6·8	6·0	6·8	1·6
2. iii. 33	6·8	6·0	7·2	0·8
27. iii. 33	8·4	—	8·8	3·2
Averages	7·7	6·6	7·9	2·7

* All four groups on control ration.

The values are shown graphically in Fig. 4. The effect of the inclusion of a foodstuff rich in carotene is again apparent in the total yellow colour of the butter. The two A.I.V. fodders show some advantage in this respect over the fodder treated with a weaker acid mixture (fed to group F2) but in this case the fodder was made from grass of poorer quality than the other two types.

The carotene contents of the three fodders used are given in Table V; they account for the somewhat smaller effect obtained with the fodder made with a lower addition of acid. The supply of this latter fodder was exhausted in early March, and this group was then discontinued.

Table V. *Carotene contents of fodders fed to the groups of Ayrshire cows.*

(Stated as mg./100 g. of the dry matter.)

Date	F 1	F 2	F 3
13. xii. 32	56·7	33·3	64·6
11. i. 33	53·9	29·5	67·2
24. i. 33	50·4	25·2	56·1
9. ii. 33	38·5	47·4	42·8
21. ii. 33	28·8	35·6	59·5
21. iii. 33	41·4	—	58·2
Averages	44·9	34·2	58·1

An interesting point is the difference in level of the maximum yellow colour value of the butter in the Ayrshire groups as compared with the Shorthorns. With the former breed it scarcely exceeded 9 Lovibond units, whereas with the Shorthorns it rose to almost 15.

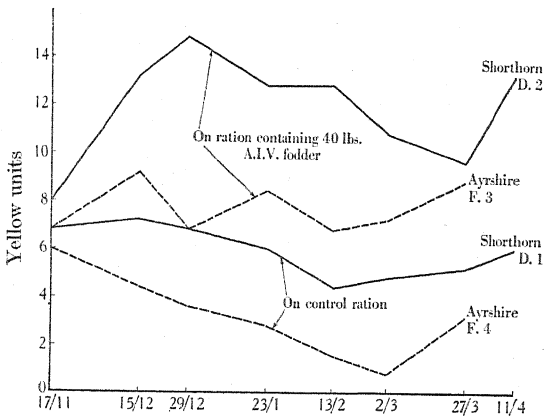

Fig. 5.

This difference with breed is shown clearly in Fig. 5, where the values for the two control groups, Shorthorn and Ayrshire, are plotted together with the values obtained when 40 lb. of A.I.V. fodder were fed (groups D2 and F3). It is noticeable that the Ayrshire butter lost its colour more rapidly and more completely than the Shorthorn on the same normal winter ration.

When a foodstuff rich in carotene was included in the ration the yellow colour of the butter was increased, but to a far greater extent with the Shorthorn cows. It seems that there is, for each breed, a "ceiling" value above which the depth of colour of the butter fat does not rise even when an excess of fodder rich in carotene is fed.

This ceiling value for the Ayrshire cows seems to be markedly lower than it is for the Shorthorns. Values obtained on the farm herds in June 1933, when the pasture was at its best, gave values of 15·2 Lovibond units for Shorthorn butter and 9·2 for Ayrshire butter, which agree well with the maximum values obtained on the A.I.V. fodder rations during the winter (14·8 and 9·2 respectively).

The difference in intensity of colour with breed has been shown by many workers, and it is generally known that the Channel Island breeds in Jersey and Guernsey produce a milk fat of higher colour than all the others, and that the Ayrshire falls below the other breeds in that regard. Wilbur *et al.* [1933] have shown that despite the difference in yellow colour, the vitamin A potency of the butter made from the milk of Jersey cows is no greater than that of Ayrshires. There are indications also from the values obtained from the butter of pure-bred Ayrshires that the vitamin A potency is similar to that of Shorthorn butter.

It would appear that the total yellow colour of butter from any one breed of cow is correlated with its carotene content and its vitamin A content, but that the depth of colour is no indication of the total vitamin A potency of the butter unless the breed from which the milk was obtained is known. A comprehensive experiment, including the major dairy breeds, is being conducted at present to obtain full data on this point.

Although the experiments were not designed to measure the effect of the various rations on the yield of milk, all yields were normal and the performance of all the cows was satisfactory.

Two trials of a different type were carried out at outside centres, the first at Dartington Hall in Devonshire[1], the other in Derbyshire. Thirteen South Devon cows were used at the former centre and eight Shorthorn cows in Derbyshire. All the cows at each centre were fed on a normal winter ration of concentrates for 5 weeks. They were all then changed over to an experimental ration containing 40 lb. of A.I.V. fodder per head daily, the nutritive value of the diet being the same as in the first period. After 5 weeks on this diet they were again changed back to the control ration, which was identical with that fed in the first period. The final period also lasted 5 weeks. The milk was sampled in the fifth week of each period, and butter samples were made and their total yellow colour was measured (Table VI).

Table VI. *Total yellow colour of the butter produced by two groups of cows on a normal winter ration and on one containing 40 lb. of A.I.V. fodder.*

Stated in Lovibond units.

	Period 1 Control	Period 2 A.I.V. fodder	Period 3 Control
South Devon cows	4·0	8·4	2·8
Shorthorn cows	2·0	8·4	2·4

The effect of the A.I.V. fodder on the total yellow colour of the butter is striking. The colour value is not so high as that obtained on 40 lb. of the A.I.V. fodder at Jealotts Hill, but it must be remembered that the experimental ration was fed for only 5 weeks and that the cows had been on a winter diet for 5 weeks, which had reduced the colour to a low level. It is a point of great practical interest that it is possible to raise the yellow colour of the milk fat so rapidly from a low level. The return of the colour to its previous low level

[1] A full account of this trial is being published elsewhere.

after reverting to the control ration for 5 weeks is indicative of the necessity for including a foodstuff rich in carotene in the diet of the dairy cow throughout the whole winter period if the colour of the milk and its quality are to be held at the high level which is so desirable from a nutritional standpoint.

II. THE CAROTENOID AND VITAMIN A CONTENTS OF THE BUTTER.

The preceding section of this paper describes the results obtained using as criterion the colour of the butter produced by the various experimental groups. The present section, on the other hand, gives the results of the spectroscopic examination of certain of the butters, with particular reference to the carotene, xanthophyll and vitamin A contents, determined by the method developed earlier [Gillam *et al.*, 1933]. The numerical results seen in Table VII correspond to the experiment described on p. 1077 (*cf.* also Table II).

Table VII.

				Constituents as mg./100 g. of dry butter			
Group	Ration	Date of milk sample	H_2O in butter	Carotene (1900 basis)	Xantho-phyll (1700 basis)	Vit. A by gross absorption 328 $m\mu$ (1600 basis)	Vit. A by absorption at 585 $m\mu$ in $SbCl_3$ colour test (2600 basis)
D 1	Control	16. i. 33	18·5	0·040	0·0186	0·292	0·222
		6. ii. 33	13·9	0·146	0·0144	0·298	0·157
		27. ii. 33	12·6	0·164	0·009	0·286	0·122
		20. iii. 33	17·9	0·025	0·0036	0·268	0·118
D 2	40 lb. A.I.V. fodder per cow per day	17. i. 33	16·0	0·607	0·065	0·63	0·480
		7. ii. 33	9·5	0·188	0·023	0·519	0·259
		28. ii. 33	12·7	0·347	0·039	0·494	0·196
		21. iii. 33	17·8	0·164	0·0134	0·407	0·281
		11. iv. 33	16·0	0·170	0·014	0·715	0·293
D 3	70 lb. A.I.V. fodder per cow per day	18. i. 33	12·8	0·543	0·063	0·372	0·294
		8. ii. 33	13·9	0·448	0·022	0·428	0·194
		1. iii. 33	17·0	0·325	0·0114	0·482	0·270
		22. iii. 33	19·1	—	0·026	0·430	0·169
		12. iv. 33	17·2	0·152	0·012	0·693	0·333
D 4	70 lb. A.I.V. fodder + 4 lb. dried grass per cow per day	18. i. 33	14·3	0·638	0·029	0·57	0·382
		9. ii. 33	12·7	0·362	0·043	0·575	0·341
		2. iii. 33	11·4	0·386	—	0·442	0·207
		22. iii. 33	12·4	0·388	0·0314	0·592	0·385
		13. iv. 33	15·6	—	—	0·578	0·241

The figures stated as the basis of calculation indicate the value of $E^{1\%}_{1\,cm.}$ taken as that of each of the pure substances. ($E = \log I_0/I$ for a 1 cm. layer of a 1 % solution, where I_0 = the intensity of the incident light and I = the intensity of the transmitted light at the wave-length of maximum absorption characteristic of each substance.)

The mean values for each of the groups over the whole period of the experiment are shown in Table VIII and clearly demonstrate the effect of the diet rich in carotene on the vitamin A and carotene contents of the butters.

A further experiment to test the value of A.I.V. fodder was carried out at Dartington Hall (Devonshire) using a single group of 13 pure-bred South Devon

Table VIII.

	Carotene mg./100 g. dry weight	Xanthophyll mg./100 g. dry weight	Vitamin A mg./100 g. dry weight U.V. values
D 1. Control	0·094	0·0114	0·286
D 2. 40 lb. A.I.V.	0·295	0·0308	0·553
D 3. 70 lb. A.I.V.	0·367	0·027	0·481
D 4. 70 lb. A.I.V. + 4 lb. dried grass	0·428	0·034	0·551

cows (see p. 1081). The results of the spectroscopic determinations are given in Table IX and, together with the evidence of the total yellow colour values given earlier, leave no doubt as to the value of A.I.V. fodder in the diet of the cow as a means of increasing the carotenoid and vitamin A contents of winter butter.

Table IX.

	First period	Second period	Third period
Ration	Control (5 weeks)	A.I.V. fodder (5 weeks)	Control (5 weeks)
Date of sample	25. i. 33	1. iii. 33	7. iv. 33
H_2O, %	13·7	12·1	12·6
Carotene, mg./100 g. of dry wt. (1900 basis)	0·122	0·311	0·129
Xanthophyll, mg./100 g. of dry wt. (1700 basis)	0·0081	0·0366	0·0148
Vitamin A, mg./100 g. of dry wt. By 328 $m\mu$ band (1600 basis)	0·183	0·405	0·240

The colour of cow's butter is known to vary with different breeds, but as the absence of yellow colour (or carotene) from butter does not necessarily imply the absence of the true vitamin A also, it was decided to examine a series of goat butters—which are noted for their chalky-white colour and low carotenoid content. Monthly samples were therefore obtained from one particular herd over the period June-December inclusive. The unsaponifiable matter was a much paler yellow than that of cow butter, and in chloroform solution it exhibited ill-defined maxima near 480 and 455 $m\mu$. As only a small fraction of the already weak yellow colour was extractable from light petroleum solution by methyl alcohol the amount of xanthophyll present must have been very small [Willstätter and Stoll, 1913]. The yellow colour remaining in the petroleum showed ill-defined absorption bands identical with those characteristic of the carotene fraction of cow's butter.

With antimony trichloride in chloroform the unsaponifiable matter gave a deep blue colour, much stronger than that to be expected from the small content of carotenoids. The blue solution exhibited two maxima at 612–618 $m\mu$ and 580–586 $m\mu$ characteristic of vitamin A, the presence of which was confirmed by the occurrence of an inflexion in the absorption curve of the chloroform solution near 328 $m\mu$.

Monthly samples (250 g.) were examined by the method applied to the cow's butter and the numerical results are given in Table X.

Vitamin A with antimony trichloride gives a blue colour exhibiting a strong absorption band at 617 $m\mu$ and a masked maximum close to 583 $m\mu$. The relative intensities of these bands are approximately 2 : 1 [Heilbron *et al.*, 1932; Carr and Jewell, 1933]. In cod-liver oil, however, the relative intensity of the 617 $m\mu$ band is considerably reduced presumably owing to the presence of certain inhibiting substances [Norris and Church, 1930]. Artificial inhibitors of this type, mostly decomposition products of proteins, have been described by

Table X. *Goat's butter.*

Date	H_2O %	Constituents expressed as mg./100 g. dry butter: Carotene* by light absorption at 460 $m\mu$	Vitamin A by 328 $m\mu$ band (1600 basis)	Vitamin A by 585 $m\mu$ band (in $SbCl_3$ blue) (2600 basis)
June	14·5	—	0·239	0·22
July	14·9	0·017	0·564	0·43
August	12·0	0·026	0·454	0·27
September	12·9	0·014	0·393	0·30
October	13·9	0·012	0·341	0·33
November	11·6	0·023	0·420	0·34
December	13·1	0·036	0·450	0·30

* Carotene values uncorrected for xanthophyll.

Table XI. *Comparison of goat's and cow's (pure-bred Shorthorn) butter.*

	Constituents expressed as mg./100 g. dry butter: Carotene	Vitamin A
Goat's butter. Mean values (June-December)	0·021	0·41
Cow's butter. Cows on ordinary winter diet (typical values)	0·15	0·28
Cow's butter. Cows on rich winter diet (typical values)	0·35	0·55
Cow's butter. Cows at grass (moderate summer values)	0·60	0·70

Emmerie *et al.* [1931], and the action of one of them—7-methylindole—on the vitamin A-antimony chloride blue colour has been examined fully [Morton, 1932]. The unsaponifiable matter of cow's butter must also contain these or similar inhibiting substances for with antimony trichloride in chloroform the intensity of the 617 $m\mu$ band is only the same or slightly more than that of the 583 $m\mu$ band and even the intensity of the latter band is reduced. This is also shown by the fact that the vitamin A values calculated from the intensity of this band are much lower than those obtained by the ultra-violet absorption at 328 $m\mu$. The distinct nature of this inhibition will be seen by a comparison of the vitamin A values measured by the two methods (Table VII). The inhibition of these absorption bands in the blue antimony trichloride solution of the unsaponifiable matter of butter is probably due to the same cause as that which gives the low blue colour values (Lovibond) on butter fat reported by Booth *et al.* [1933], who find the amount of inhibition to vary seasonally. In the samples of the unsaponifiable matter of goat's butter we find that although both absorption bands are reduced in intensity the inhibition is not so marked as in the case of cow's butter. This is shown by the fact that the vitamin A values of goat's butter, calculated from the intensity of the 585 $m\mu$ absorption band, do not differ quite so much as in the case of cow's butter from those obtained by the ultra-violet absorption method (Table X).

Summary.

The effect of a ration containing artificially dried grass on the quality of the milk of Shorthorn cows was tested. When 10 or 25 % of the concentrated foodstuffs fed in the production ration were replaced by artificially dried grass no appreciable advantage in depth of yellow colour of the butter-fat was noted, as compared with a control group on a normal winter ration. The inclusion of

50 % of artificially dried grass in the concentrated ration, however, caused a distinct improvement in the yellow colour of the butter fat.

The inclusion of A.I.V. fodder in the winter ration of Shorthorn cows raised the yellow colour of the butter-fat to a high level, comparable with that obtained on best pasture. The A.I.V. fodder was fed at two levels, 40 lb. and 70 lb. per head daily. The larger amount showed no advantage, either in colour or vitamin A content of butter fat, over an allowance of 40 lb. per head daily.

The feeding of A.I.V. fodder increased the carotene and xanthophyll contents of the resulting butter to about three times those of a similar control group on a normal winter ration. The content of the true vitamin A was approximately doubled at the same time.

The feeding of A.I.V. fodder to Ayrshire cows raised the yellow colour of the butter-fat to a level well above that from milk produced by a group on a normal winter ration.

The difference in the depth of yellow colour of butter fat as between breeds, is well marked in the case of Shorthorn and Ayrshire cows. On a normal winter ration deficient in carotene, the yellow colour of the butter fat of Ayrshire cows fell more rapidly and to a decidedly lower level than was the case with Shorthorn cows. There seems to be, for each of these two breeds, a "ceiling" value above which the colour intensity and vitamin A content of the butter fat do not rise, even when an excess of carotene-rich fodder is fed.

The feeding of 40 lb. of A.I.V. fodder in the ration raised the yellow colour of the butter fat of Shorthorn cows to a high "ceiling" level, whereas with the Ayrshire the "ceiling" value was much lower, though the amount of carotene supplied in the ration was similar for both breeds. In spite of this difference in depth of colour, it appears that there may be little difference in the content of true vitamin A in the butters from the two breeds.

Samples of goat's butter were found to be very similar to cow's butter in their content of the true vitamin A, although they were almost devoid of colour; the carotene content was less than 5 % of that of the butter from cows at grass. Thus the colour of goat's butter cannot be used as an indication of its growth-promoting activity in comparison with cow's butter, since in goat's butter most of this activity is due to the presence of colourless preformed vitamin A and not to carotene.

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REFERENCES.

Booth, Kon, Dann and Moore (1933). *Biochem. J.* **27**, 1189.
Carr and Jewell (1933). *Nature*, **131**, 92.
Emmerie, Eekelen and Wolff (1931). *Nature*, **128**, 495.
Gillam, Heilbron, Morton, Bishop and Drummond (1933). *Biochem. J.* **27**, 878.
Heilbron, Heslop, Morton, Drummond, Rea and Webster (1932). *Biochem. J.* **26**, 1178.
Morton (1932). *Biochem. J.* **26**, 1197.
Norris and Church (1930). *J. Biol. Chem.* **85**, 477; **87**, 139.
Virtanen (1933). *Emp. J. Exp. Agric.* **1**, 143.
Watson, Drummond, Heilbron and Morton (1933). *Emp. J. Exp. Agric.* **1**, 68.
Willstätter and Stoll (1913). Ueber Chlorophyll. (Berlin.)
Wilbur, Hilton and Hauge (1933). *J. Dairy Sci.* **16**, 153.

CXLVIII. AN ANALYTICAL STUDY OF LIESEGANG RINGS.

By EDWIN BURNHOPE HUGHES.

(*Received March 12th, 1934.*)

So much has been written on Liesegang rings, and so many theories or variants of theories proposed, that apology may be expected for further addition, though perhaps the investigation of the phenomenon mainly by the application of quantitative analysis may be regarded as some justification. The literature of this subject has been liberally referred to in most of the papers published thereon in recent years, including an exceptional comprehensive bibliography by Hedges [1932] and it is accordingly considered unnecessary to repeat such references here, except in those instances where specific mention is essential.

In order to make clear the trend of the results of this investigation, it may be stated that they lead to the conclusion that, when diffusion of one electrolyte into a solution of another of lower equivalent concentration (generally contained in a gel) causes formation of a precipitate, the occurrence of this precipitate in band formation depends essentially on the following factors:

(1) The gradient of the concentration of the entering diffusing reacting salt (precipitating ion, A) and the resulting gradient, at the precipitate front, of the concentration of the reacting ion (B) contained in the medium; and on the removal of ion B in the neighbourhood of the precipitate by the advent of more of ion A till forward diffusion of electrolyte A again proceeds normally.

(2) On the rapid collection of the separated precipitate into a more closely defined zone than when first separated.

(3) On the solubility of the precipitate and on some factor explaining sudden separation of precipitate, which is taken to be metastable solubility.

Factors (2) and (3) are essential conditions to the extent that on them depend the character of precipitate and the conditions for (1) requisite to control the formation of the precipitate in banded form or as continuous precipitate.

Furthermore, the primary importance of the movement of the electrolytes towards each other is shown by the fact that banded precipitation can be obtained when this movement of ions is produced under the influence of a superimposed E.M.F., *i.e.* by the application of electrolysis [Hughes, 1928].

None of these factors is new in its application to the explanation of the formation of Liesegang rings; they have been proposed and referred to, to a greater or less extent, in many notable papers, but in this communication they are submitted as a conclusion from the experimental evidence recorded here. There are other factors which may be of importance in certain instances only—such as adsorption, and coagulation of the precipitate by the entering electrolyte (or one of its ions)—but these factors are to be regarded as of minor importance only and not as essential causes of banded formation of precipitate.

A theory of the formation of Liesegang rings follows the description of the experimental work.

A. Diffusion.

The process of diffusion of an electrolyte into a gel follows Fick's law of diffusion, and the mathematical expressions for the concentration gradient and the rate of penetration have been developed and verified experimentally by a number of workers, *e.g.* Morse and Pierce [1903], Stiles [1920], Stiles and Adair [1921], Adair [1920]. A very valuable mathematical and theoretical study of diffusion in respect of rhythmic precipitates is that of Fricke [1923]. The effect of temperature on rate of diffusion is considerable: Stiles [1921] states that increase of temperature from 0° to 20° increases the diffusion coefficient about 1·76 to 2·2 times, according to the salt.

A I. *The passage of water into or from the gel.*

The possibility of the movement of water into the gel from the outside or *vice versa* is generally ignored though, for example, Hatschek [1922], Davies [1922] and Steopoe [1924], have suggested that alteration of the water content of the gel may have some bearing on the formation of Liesegang rings. Accordingly it seemed worth while, first of all, to ascertain if such action takes place to an appreciable extent.

Diffusion was caused to take place for about 100 hours from a weighed solution of a salt of known concentration into a weighed tube of gelatin gel, after which the solution and gel were separately weighed and analysed for salt in the solution and salt and gelatin solids in the gel. Similar tests were made with a salt in the gel diffusing out into the water. The relative amounts of aqueous solution and of gel were varied. The materials used were: NaCl solution and gelatin gel and agar gel; K_2CrO_4, also $AgNO_3$ into plain gelatin gel and into the gel containing the other salt; water into gelatin gel containing NaCl. Results showed that the movement of water only occurred to a negligible extent, generally about 0·1 to 0·3 g., being to the solution of higher osmotic pressure, and always only from near the surface of the gel. It is concluded that, in connection with diffusion from solutions into gels, the movement of water is very slight and is negligible.

Actually if two reacting salts are separately contained in gels in contact, diffusion from one gel to the other with formation of a banded precipitate can occur.

A II. *Analysis, with respect to the entering electrolyte, of gels in which diffusion has taken place with formation of precipitate (continuous or banded).*

In general, a quantity of standardised solution of one electrolyte was mixed with liquefied gel of concentration double that finally required and of half the required volume, and the mixture then made up to volume with distilled water. A portion was taken for analysis and the remainder filled into corked glass tubes of about 2·5 cm. diameter and suitable length. When the gel had set firm, it was attached by rubber tubing to the neck of a flask (300 ml.)[1] containing the intra-diffusing electrolyte, and then placed vertically, with flask inverted, and the diffusion allowed to proceed at constant temperature to the required extent[2]. The flask was then disconnected,

[1] In general sufficient volume of reagent to ensure only the slightest diminution of concentration during diffusion.

[2] It was proved that this method gave entirely satisfactory results, and that there was no entry of the diffusing liquid itself in experiments recorded here.

With gelatin gels or silicic acid gels no entry of diffusing liquid ever occurred; with agar gels it was found that this trouble could be avoided by filling the hot agar solution (at about 95°) into hot tubes taken direct from a steam-oven, the contraction of the agar from the glass, on setting, being thus avoided.

free liquid carefully removed from the surface of the gel with filter-paper, and the gel removed from the tube by one of the following methods.

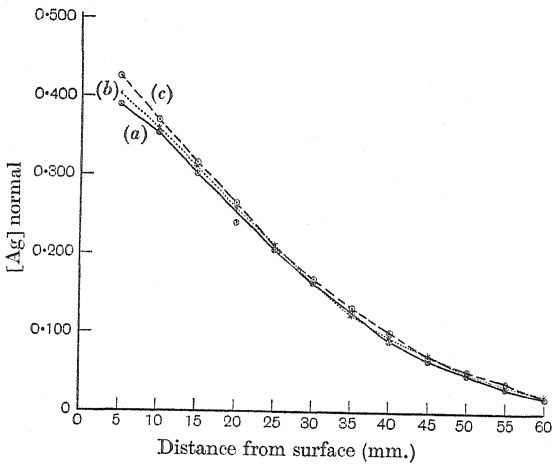
(*a*) The tube used had been first carefully split in two, the halves bound together with rubber bands and the cut edges waxed over before filling. Afterwards, when the gel was required for analysis, the wax was scraped off the tube and the two portions carefully separated, leaving generally a clean undamaged cylinder of gel.

(*b*) Procedure (*a*) was not always successful; occasionally a gelatin gel would adhere to the tube in places, spoiling the experiment. The method finally adopted, which avoided the use of split tubes, was to remove the gel by gently pushing a long stiff thin wire along the inside of the tube, closely in contact with the glass, and then, while one person held the wire firmly, another gently rotated the tube so that the wire was always against the glass. The gel was so loosened and could then be blown out of the tube.

(*c*) Agar gels contract on setting, so that all that is necessary is to cut round the top edge of the gels (where contact had purposely been made sound to avoid entry of diffusing liquid) and then to blow the gel out of the tube.

The gel was then sliced with a razor blade into portions of suitable thinness, and these were placed at once in small covered weighed beakers and weighed, and the slices were then analysed. Generally the thickness of each slice was not measured directly, but calculated from its weight in relation to the total weight of the slices, the total length of the gel before cutting being noted. Distances were calculated as being to the middles of slices, and in recording analytical results these are given as if the slices were of uniform composition. In the graphs the concentration of reagent in a slice is given as at the middle point, or sometimes shown (for bands and clear spaces) as being equal throughout the slice.

Graph 1 (curves *a*, *b*, *c*) shows the concentration of silver in the gel after diffusion thereinto of $N/2$ $AgNO_3$ solution (*a*) into plain 5 % gelatin gel; (*b*) similar gel containing $N/500$ K_2CrO_4; (*c*) gel containing $N/500$ Ag_2CrO_4.



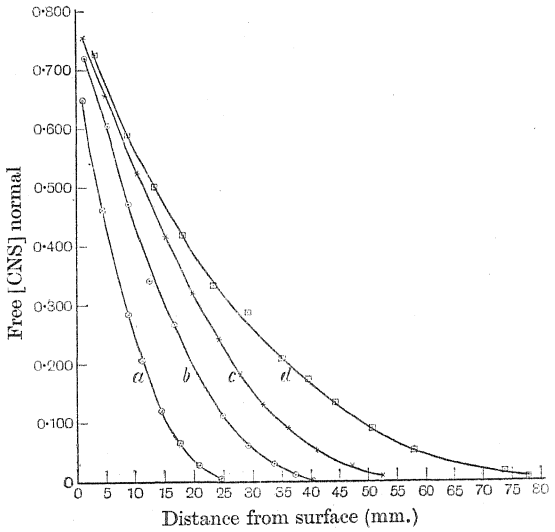
Graph 1.

(*a*) ⊗——⊗ 5 % gel only. (*b*) ×----× 5 % gel + $N/500$ K_2CrO_4.
(*c*) ⊙---⊙ 5 % gel + $N/500$ Ag_2CrO_4.

These results show that in presence of Ag_2CrO_4 in solution or of very dilute K_2CrO_4 in the gel, the concentration curve of the intra-diffused $AgNO_3$ is of the same type as for the diffusion into the plain gel. (*b*) and (*c*) show slightly higher concentration of Ag than (*a*) due to the Ag precipitated by the K_2CrO_4 and to the Ag_2CrO_4—but the amount of Ag so held is not constant along the tube,

i.e. is not always $N/500$, this being due to some movement of the contained K_2CrO_4 (and Ag_2CrO_4) towards the entering silver. This effect is not very noticeable for such dilute K_2CrO_4 and, moreover, the rings are not shown separately; clear evidence of this movement is given in results described later.

The inference, from the deduction and observation of Morse and Pierce [1903] that the relationship $d/\sqrt{t}$ applies during ring-formation, is that the diffusion proceeds in accordance with Fick's law all through the precipitate formed, and that the concentration of the uncombined entering diffusing ion should therefore give an ordinary diffusion curve. Results such as those shown in Graph 1, comparing diffusion in absence and in presence of a precipitating ion, indicate that this is the case. Consequently, subtraction of the concentration of other ion (contained in the gel) from the equivalent of the concentration of diffusing ion at the same point should give a regular diffusion curve for the differences so obtained, *i.e.* for the "free" ion[1]. This procedure has been followed in considering the analytical results which follow. A simple case is where the "free" ion can be determined directly without having to determine each of the reacting ions and subtract; for example, Graph 2 (curves *a*, *b*, *c* and *d*), which expresses the results of analysis after diffusion of ammonium thiocyanate into 5 % gelatin containing $N/70$ silver nitrate for 19, 43, 67 and 139 hours. The tubes and amounts of reagents were identical, so that the results can be taken to represent the concentration in one tube after these different periods of diffusion.



Graph 2.

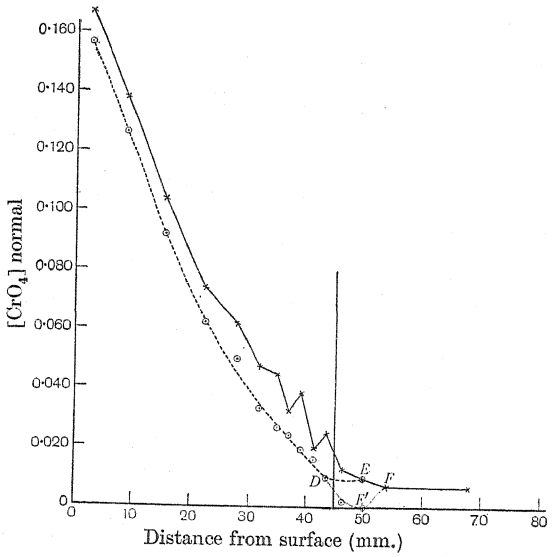
a After diffusion for 19 hours. *b* After diffusion for 43 hours.
c „ „ 67 „ *d* „ „ 139 „

It will be seen that the curves, which are for "free" [CNS], are satisfactory diffusion curves. They also show clearly the flattening of the gradient as diffusion proceeds further. The results obtained for [Ag] (not shown) indicate that there is also a diffusion gradient of the [Ag] in the gel towards the precipitate front.

[1] The term "free," in considering the ion or the salt, is applied to the amount or concentration of it which is not combined or associated with the other ion with which it forms a precipitate.

When separate bands and "clear" spaces are analysed, results have to be interpreted as to the centre of the bands, and to inexactness from this must also be added some inaccuracy resulting from not being able to cut slices exactly to the edge of a band, even if sharply defined, and also that resulting from the double experimental error which may be involved in subtraction. Nevertheless, the results obtained[1] provide satisfactory evidence of the regular diffusion of the "free" ion. Examples are given in Graphs 3, 4, 5 and 6.

Diffusion of M/*10* K_2CrO_4 *into 5 °/₀ gelatin containing* N/*100* $AgNO_3$. *Analysis after diffusion for 121 hours* (Graph 3). The general form of the curve for "free" $[CrO_4]$ is in accordance with diffusion, as expected. The "free" curve is joined



Graph 3.

× —— × Total $[CrO_4]$. ⊙- - - ⊙ Free $[CrO_4]$.

to the total curve at E because from there onwards the CrO_4 is in presence of Ag without any precipitate having been formed. $DE'F$ would represent the conditions when precipitation for a band occurs at (or about) E'.

Diffusion of M/*5* $K_2Cr_2O_7$ *into* N/*100* $AgNO_3$ *in 5 °/₀ gelatin* (Graph 4). Analysis was made of separate bands and spaces from 103 mm. below the surface, where the bands were wide enough apart for separation. The last ring was incompletely formed. The time of diffusion was 52 days, after which the action had practically come to a standstill although there was abundant $AgNO_3$ remaining, this having been assured by using a long tube of gel fitting into a flask of the gel.

With $AgNO_3$ as the internal electrolyte the diffusion of $K_2Cr_2O_7$ into the gel results in the formation of Ag_2CrO_4[2] (not $Ag_2Cr_2O_7$), according to the equation

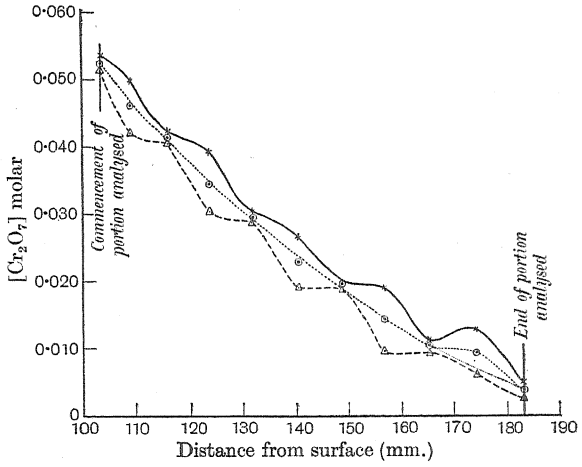
$$H_2O+K_2Cr_2O_7+4AgNO_3=2Ag_2CrO_4+2KNO_3+2HNO_3.$$

The "free" $[Cr_2O_7]$ is calculated accordingly, and the curve is seen to be of a satisfactory character, except towards the end of diffusion; here, however, the

[1] In none of these examples is allowance made for the solubility of the precipitate; such allowance would not affect the shape of the "free" curve.

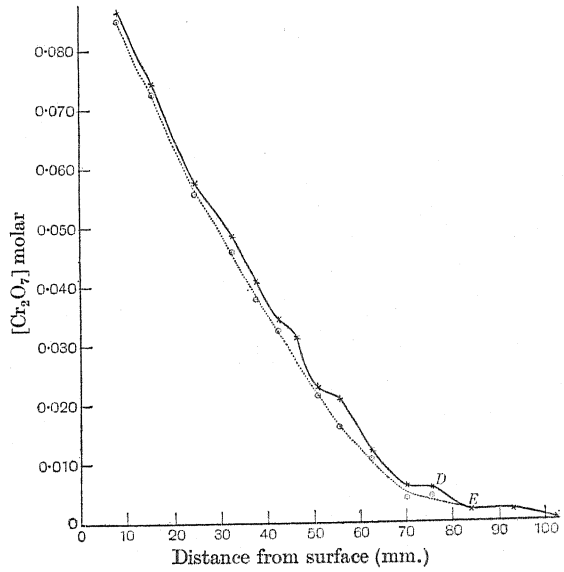
[2] Davies [1922], also confirmed (in addition to Graph 4) by tests applying Pringsheim's rule and by the colour and nature of the precipitate.

last ring was ill-formed and indefinite, there being some other separation of precipitate, near the band, on the glass tube. For comparison, the curve for "free" $[Cr_2O_7]$ assuming formation of $Ag_2Cr_2O_7$, is also given. $K_2Cr_2O_7$ is more suitable than K_2CrO_4 for obtaining bands for analysis, as the spacing is wider; this can be explained by the lower effective concentration of the $AgNO_3$ when $K_2Cr_2O_7$ is used with it.



Graph 4.

× —— × Total $[Cr_2O_7]$. ⊙- - - -⊙ Free $[Cr_2O_7]$ (formation of Ag_2CrO_4).
△– – –△ Free $[Cr_2O_7]$ (if $Ag_2Cr_2O_7$ were formed).



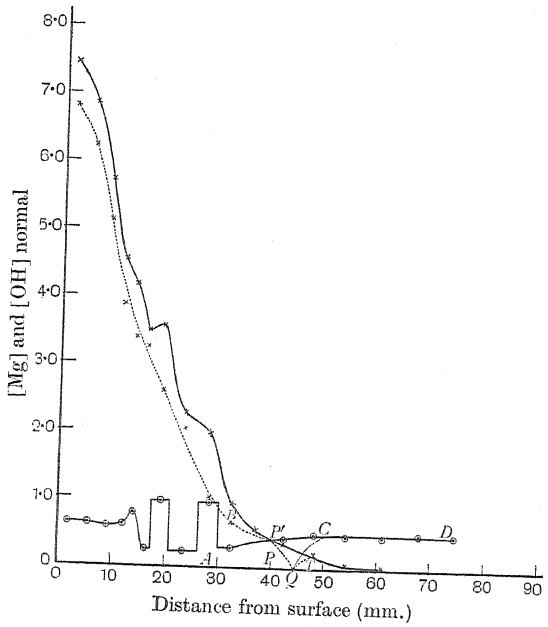
Graph 5.

× —— × Total $[Cr_2O_7]$. ⊙- - - -⊙ Free $[Cr_2O_7]$.

Diffusion of 0·1 M $K_2Cr_2O_7$ for 257 hours into 0·0075 N $AgNO_3$ (another example of the same type) (Graph 5). The "free" $[Cr_2O_7]$ curve is run into

the total [Cr_2O_7] curve between points D and E for the same reason as stated with reference to Graph 3. The gradient of the concentration of the Ag has become very flat from E onwards, and progress of diffusion of both $K_2Cr_2O_7$ and Ag has practically ceased (as occurs relatively early when a salt in dilute solution diffuses, with formation of precipitate, into one very dilute).

Diffusion of ammonia (solution of sp.gr. 0·880) into N/2 $MgCl_2$ contained in 1 % agar gel. Analysis after diffusion for 48 hours (Graph 6). At the moment of cutting the gel the last actual precipitate was at A. The "free" [OH] curve should then pass on to the total [OH] curve at P' after which OH is "free" in the presence of Mg. Apparently at about PP', say at Q, precipitation is due. The "free" [OH] should there drop to a low value as BQT; "free" [Mg] would drop from P' to a low value at Q, so that its curve would have a gradient from Q into CD, say at C.



Graph 6.

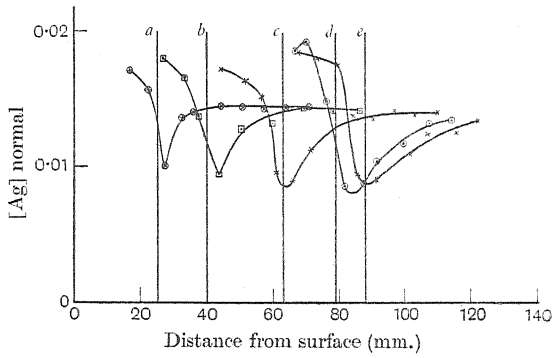
× —— × Total [OH]. ⊙ —— ⊙ Total [Mg]. × - - - - × Free [OH]. × ..... × Free [Mg].

In the examples just given it will be noted that the entering ion penetrates some distance into the gel beyond the last precipitate (band) without forming precipitate. This distance of penetration obviously has relation to the spacing of the bands; it is, in fact, a governing condition. The flatter the curve the further will the ion penetrate without forming precipitate; accordingly a concentrated solution (of the entering ion) will tend to give continuous precipitate, as, its gradient being steep, it will have little penetration beyond the precipitate front, and a dilute solution will give bands—if other conditions are also suitable. When the concentration curve of a concentrated solution becomes sufficiently flat owing to time and distance of diffusion, then the precipitate may change from continuous to band-formation.

The concentration of the ion contained in the gel also has its effect on band-formation, and accordingly the gradient of the concentration of the internal ion has been examined.

A III. *Analysis, with respect to the internal electrolyte, after diffusion has taken place with formation of precipitate (continuous or banded).*

Curves *a*, *b*, *c*, *d* and *e* in Graph 7 show results of analyses of the gel in the neighbourhood of the precipitate front after diffusion of *N* K_2CrO_4 into 0·0143 *N* $AgNO_3$ in 5 % gelatin in similar tubes for different periods (16, 42, 112, 161, 209 hours). These conditions give close but separate bands[1].



Graph 7.

The vertical lines represent the precipitate front (as nearly as it could be cut) for the tubes *a*, *b*, *c*, *d*, *e*. The results show clearly:

(1) that the Ag, in amount, has advanced very considerably towards the incoming K_2CrO_4. This is to be expected from the gradient of the [Ag] at the precipitate front, down which the Ag must move.

(2) The gradient flattens with the age (and distance) of the diffusion of K_2CrO_4. (It is the right-hand slope of the curve which is to be considered.) This change in the gradient of the internal electrolyte results in the widening of spaces between rings; it is dependent on the rate of diffusion of the entering ion. If the conditions of diffusion give continuous precipitate, or rings so closely arranged as to be merely denser zones with precipitate between, then the gradient of the contained ion shows but little alteration as diffusion proceeds.

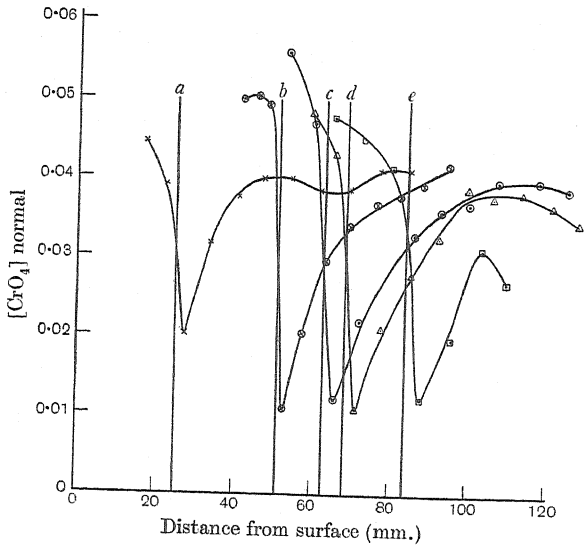
Curves *a*, *b*, *c*, *d* and *e* in Graph 8 illustrate this in the case of diffusion of 0·5 *N* $AgNO_3$ into 0·04 *N* K_2CrO_4 in 5 % gelatin for 24, 120, 172, 216, 312 hours. In tube *e* the CrO_4 remaining uncombined is much reduced in amount.

Graphs 9 and 10 are of interest as showing results when either concentrated $AgNO_3$ or K_2CrO_4 diffuses into the other also rather concentrated. Such conditions result in continuous precipitate.

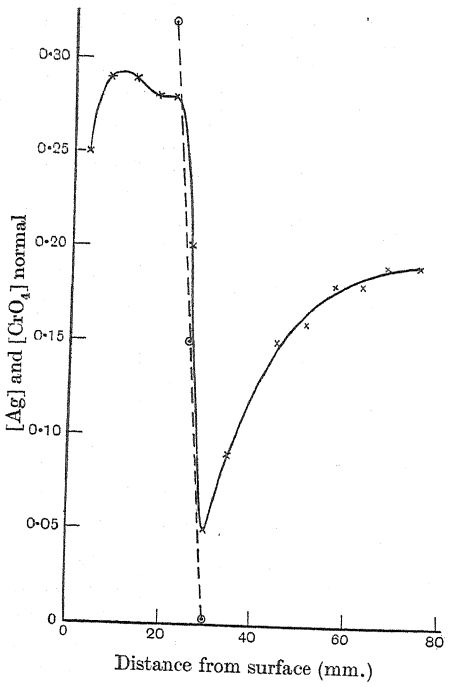
The observations of interest are:

(1) The steepness of the curve of the concentration of the entering electrolyte. This is a condition for formation of continuous precipitate. This steepness is due, first to the high concentration of the outside electrolyte and, secondly, to the somewhat high concentration of the internal electrolyte which has the

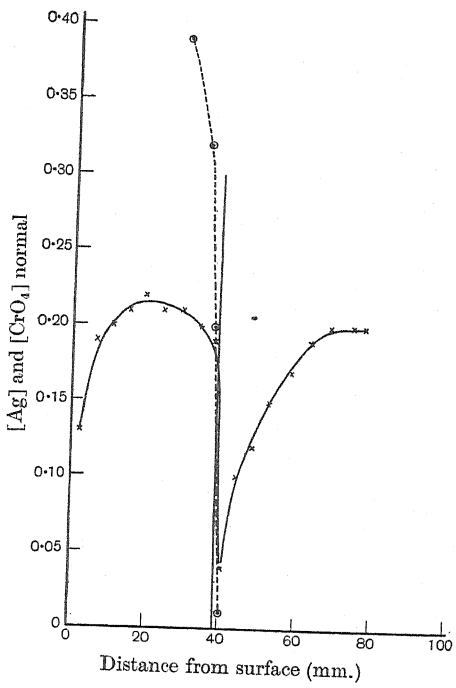
[1] For the purpose of obtaining clear spaces and bands of Ag_2CrO_4 for analysis, it is preferable to have $AgNO_3$ as the internal electrolyte.



Graph 8.



Graph 9.



Graph 10.

Graph 9. Diffusion of N K_2CrO_4 into 0·2 N $AgNO_3$ in 5 % gelatin for 93 hours.
× —— × Total [Ag]. ⊙— —⊙ Total [CrO_4].

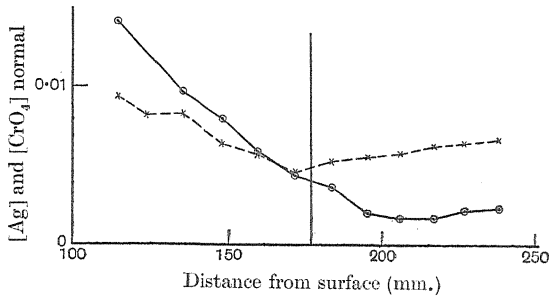
Graph 10. Diffusion of N $AgNO_3$ into 0·2 N K_2CrO_4 in 5 % gelatin for 70 hours.
× —— × Total [CrO_4]. ⊙- - - -⊙ Total [Ag].

effect of slowing down the diffusion of the entering electrolyte, *i.e.* making k, of $d/\sqrt{t}=k$, much smaller. Thus, for example, diffusion of N $AgNO_3$ into 5 % gelatin containing (*a*) no added salt, (*b*) $N/100$ K_2CrO_4, (*c*) $N/50$ K_2CrO_4, (*d*) $N/10$ K_2CrO_4, (*e*) $N/5$ K_2CrO_4, gave results as follows for periods up to 112 hours: k (cm.-hours) *a*, 0·74; *b*, 0·68; *c*, 0·64; *d*, 0·46; *e*, 0·38.

(2) The gradient of the concentration of the internal electrolyte, at the precipitate front, is also steep. The consequence is that the steepness of both gradients ensures that a very slight advance of the entering electrolyte will provide sufficient of it in the internal electrolyte to produce the amount required for the metastable solubility[1] of the precipitate, which is therefore continuous.

(3) The internal electrolyte has moved up the tube very considerably.

Graph 11 gives the results of conditions of diffusion of the extreme opposite of the last.



Graph 11.

⊙——⊙ Total $[CrO_4]$. ×---× Total [Ag].

Rather dilute K_2CrO_4 ($N/10$) had been diffusing into very dilute ($N/100$) $AgNO_3$ in 5 % gelatin contained in a long tube which was continued into a flask of the $AgNO_3$ in gel, so that there was abundance of each electrolyte. For 21 days bands continued to form, after which time diffusion had become so slow that in a further 38 days only a trace more of precipitate had formed, and this only on the wall of the tube.

The analytical results show that after a depth of 170 mm. the gradient of $[CrO_4]$ was so slight that the CrO_4 diffused for 67 mm. further without rising above 0·002 N, and that similarly the gradient of the [Ag] was so flat that even at 60 mm. from the precipitate front the concentration was only 0·006 N, hardly enough to cause precipitation. Under such conditions diffusion of both electrolytes virtually comes to a standstill, and it is probable that, as the real metastable solubility is not reached, some partial separation of precipitate occurs, for example, on the walls of the tube—as observed; this would also probably account for the slight rise in $[CrO_4]$ at the end of the diffusion.

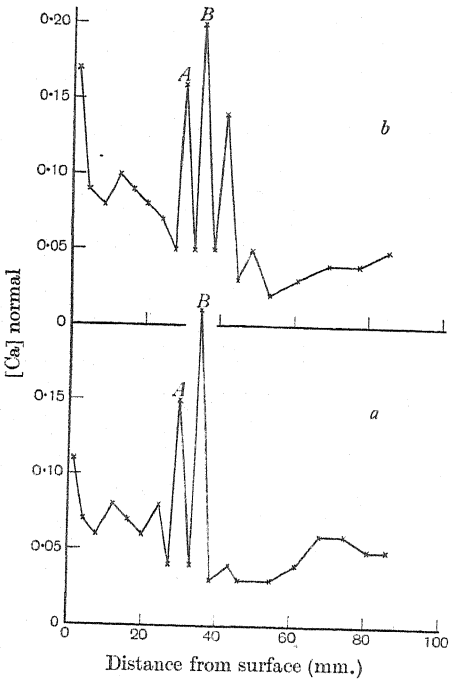
A IV. *Permanency of amount of precipitate in fully formed bands.*

The results just considered all show that the concentration of the internal electrolyte falls to a small quantity (actually, at the lowest point, the solubility of the precipitate), in the immediate neighbourhood (front) of the precipitate, and accordingly it is to be expected that this electrolyte does not pass appreciably further towards the entering ion. This implies that a band, once fully formed, does not subsequently acquire any increase in precipitate caused by

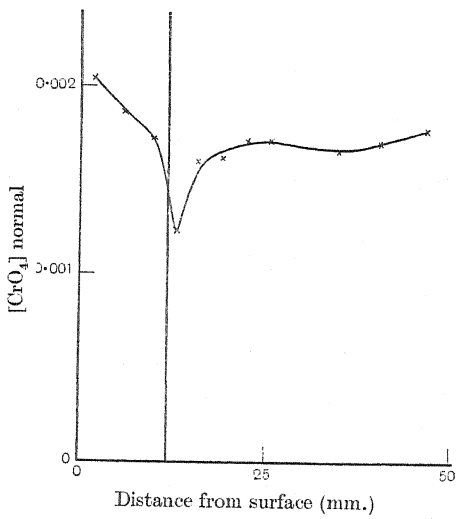
[1] See p. 1099.

the reacting electrolytes, though for some time after the formation of a band, until fully formed, there will be some considerable intensification of the precipitate forming it, due to collection on to it of precipitate from the adjacent "clear" spaces (p. 1100), and also due to the reduced solubility of the precipitate, if this is less soluble in higher concentrations of entering ion, *e.g.* Ag_2CrO_4 in $AgNO_3$; the reverse, *i.e.* increased solubility, also occurs, *e.g.* AgCNS in NH_4CNS. The following is an example showing that the bands, once completely formed, continue, in the main, unchanged in intensity.

Curves *a* and *b* in Graph 12 show the distribution of calcium in two identical tubes after diffusion of 0·166 *M* Na_2HPO_4 into a 1 % agar gel containing 0·066 *N* $CaCl_2$ for (*a*) 141 hours and (*b*) 237 hours. If one of the curves is superimposed on the other it will be seen that the peaks *A* and *B* in the one fit almost exactly on the same two peaks of the other.



Graph 12.



Graph 13.

A V. *Diffusion of silver chromate.*

A particularly interesting example of analysis after diffusion is that showing results of diffusion of a solution[1] of silver chromate contained in a gelatin gel, towards incoming silver nitrate, when bands of precipitated silver chromate are formed.

This precipitation has been described by Williams and MacKenzie [1920] and by Hedges and Henley [1928], the former considering it to be evidence of diffusion of silver chromate, and the latter as periodic coagulation or periodic salting out; both of these actions occur, but the results here given particularly

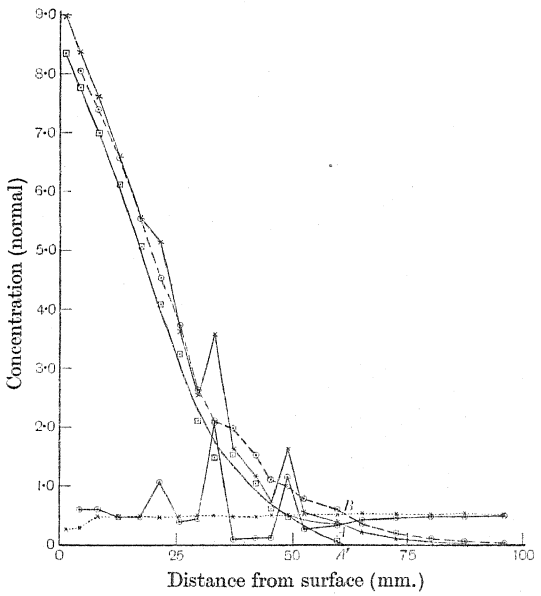
[1] Experimental evidence as to the solution-state of Ag_2CrO_4 when yellow has also been given by Bolam and Mackenzie [1926] and Desai and Nabar [1931; 1932].

confirm that silver chromate in solution does diffuse and band formation is to be attributed to this.

In this experiment, ash-free gelatin prepared by the method of Loeb [1922] was used in order to avoid the interference due to chlorides and phosphates; in such gelatin the solubility of Ag_2CrO_4, as measured by the maximum amount which still gives only a yellow colour (no tinge of red), is very low, and in consequence the concentration of Ag_2CrO_4 used was only $N/500$. Diffusion was for 4 hours only. Such a brief period was necessary in order that the gradient of the $[Ag_2CrO_4]$, which at most can only be short, shall be as steep as possible, this being governed by the rate of diffusion of the entering $AgNO_3$.

A VI. *The unprecipitated electrolyte of the reaction.*

Results so far given have been concerned solely with the precipitating ions and not with the unprecipitated product (or ions) of the reaction. A convenient example of ring-formation for this full analysis is provided by the diffusion of ammonia into magnesium chloride in agar gel which other workers [Ostwald, 1925; Hedges and Henley, 1928; Fricke and Suwelack, 1926] have found particularly suitable (Graph 14).



Graph 14.

× —— × Total [OH]. ⊙– – –⊙ Total [NH_4]. ⊙ —— ⊙ Total [Mg].
× - - - - × Total [Cl]. ⊡ —— ⊡ Free [OH].

Consider the concentration curves for each ion after diffusion for a period. The [Mg], ion contained in the gel, will give a curve having the usual gradient towards the last formed precipitate and with peaks representing the bands of $Mg(OH)_2$. The $[NH_4]$, entering ion, will give a diffusion curve representing mainly NH_4OH and some as NH_4Cl—but on the whole it should be an ordinary diffusion curve without peaks due to any local concentration. The [OH], entering ion, will give a diffusion curve almost coincident with the $[NH_4]$ curve except

for peaks showing the bands of $Mg(OH)_2$. The "free" [OH] will give a concentration curve very similar to that of the $[NH_4]$, separated only by the equivalent of the [Cl].

There remains the [Cl], ion contained in the gel. It has not entered into any real combination or diffusion. It should therefore show no peaks, and, as it was originally uniformly distributed throughout the tube it should remain so—without any diffusion, except perhaps on account of NH_4Cl when formation of $Mg(OH)_2$ takes place, which would diffuse in both directions from the zone of the reaction; there would be also some diffusion out into the NH_4OH in the reservoir. Consequently the [Cl] curve should be, in the main, a horizontal line. A typical example (diffusion of ammonia, sp. gr. 0·880, into 0·5 *N* $MgCl_2$ in 1 % agar gel), Graph 14, shows that these conditions are fulfilled.

These results, which are all analytical determinations, also indicate another matter of interest, namely that, up to the forefront of the precipitate, there is diffusion of NH_4OH as molecular diffusion, but that from that zone the diffusion becomes ionic. At the front of the precipitate the action occurring is the union of OH and Mg, with the consequence of diffusion of NH_4 forward in excess of the OH, and diffusion of Mg towards the OH, always, of course, so that $[Mg]+[NH_4]=[OH]+[Cl]$. For example, note the two points *A* and *B* where [OH] and [Mg], and $[NH_4]$ and [Cl] are respectively equal.

The point *A* has also another significance. It will be noted that the curve for "free" [OH] is joined on to that for total [OH] at the point *A*, on the assumption that, from about there onwards, all the OH is free.

Fricke and Suwelack [1926] have published analyses and curves of a number of examples of diffusion of NH_4OH into $MgCl_2$ contained in agar—chiefly from the last ring (or 2 rings) forward into the $MgCl_2$ and have shown therefrom the dependence of ring-formation on the ion activity product.

Summary of the results and conclusions from the analytical examination of banded and continuous precipitate.

1. The results recorded indicate the predominant effect of conditions of diffusion in governing the resulting form of the precipitate, *i.e.* whether it is banded or continuous. The theoretical relation of gradient of entering ion to spacing of the bands is described later.

2. They show also that the reaction is limited to the precipitate front with, as suggested by the results of microscopic examination of rapidly forming bands (as under the influence of a superimposed E.M.F.), some condensation and growth of the precipitate immediately it is liberated and some subsequent clearing of spaces due to reduction in solubility caused by the increasing concentration of the entering ion.

3. At the precipitate front is a gradient of concentration of the ion contained in the medium (gel). This gradient obviously has an effect on the spacing of bands, and is dependent on the concentration of this ion in the medium and also on the gradient of the concentration of the entering ("free") ion.

Before these conclusions can be embodied clearly in a theoretical explanation of the formation of Liesegang rings, the specification of some other conditions becomes necessary, namely some factors which will account for the sudden liberation and development of precipitate. These factors are not essential only to banded precipitate; they will function when the precipitate is continuous. The conceptions which seem best to meet these requirements are: (*a*) metastable solution and (*b*) growth of precipitate particles at the expense of the smallest

[Jablczynski *et al.* 1923], and (*c*) removal of contained ion in the zone of the precipitate (as described on p. 1103). Some brief reference is therefore given to these.

B. METASTABLE SOLUTION AND DEVELOPMENT OF PRECIPITATE.

Much of the published work with reference to production of banded precipitates is a record of the precipitates which will, under specified conditions, form in bands. It is generally agreed that almost any substance which is relatively insoluble can be obtained in band-formation if the conditions are satisfactory [see Hedges and Henley, 1928; Morse, 1930]. Notable among these conditions is the degree of solubility of the precipitate in the medium, which is generally a gel, but which may also be simple aqueous solution. The solubility can be so altered by choice of the medium or by addition of a small quantity of some foreign substance that bands can be obtained for almost any material which forms as a precipitate by double decomposition. This is well recognised [see Bradford, 1920; Morse, 1930].

Supersaturation and metastable solution.

Banded precipitates can only be formed in conditions which are actually ideal for the occurrence of supersaturation, an important condition being undisturbed diffusion (with or without a gel). In such circumstances there is none of the solid phase present where precipitation occurs, and no disturbance to cause the solubility to be limited to the true solubility. On the other hand the sparingly soluble salt formed does not remain indefinitely in solution but appears to be released at some limiting value, indicating a metastable limit. Though such an occurrence may not be capable of direct proof[1], it seems to be a necessary assumption in accounting for the formation of precipitate in bands. The arguments in favour of this opinion have been put forward by many workers, notably Morse and Pierce [1903] who state:

"We have shown by quantitative measurements that in the formation of silver chromate in gelatin solution, a definite constant product of $Ag_2CrO_4 = H$ exists which determines the limit of supersaturation for silver chromate in the absence of the solid phase."

Morse [1930] gives extensively the evidence in favour of occurrence of supersaturation and the rapid release of salt from a greatly supersaturated solution. Fricke [1923] writes:

"There is no reason for assuming a transient diminution of solubility during the formation of deposits. To do without the assumption of a more or less well-defined metastable limit in the sense of W. Ostwald, *i.e.* an ion limit product in order to explain the theory of Liesegang rings seems to be impossible."

Notboom [1923] also makes use of the idea of the metastable limit and in support quotes the development of this idea from consideration of surface energy as viewed by Haber and Vollmer.

It is in this sense of metastable limiting solubility that the term "solubility" is employed in this paper, and a limiting "solubility" product is considered as being applicable.

[1] When the formation of bands, as for example by CrO_4 entering into $AgNO_3$ in gel in a capillary tube under the influence of a superimposed E.M.F., is observed under the microscope, it is seen that the advancing CrO_4 colours the gel a faint orange, and behind the wave front of the CrO_4 a band appears as a sudden darkening of the gel increasing rapidly in intensity. By suitable choice of E.M.F. and concentration of reactants, bands can be produced at regular intervals of space and time (about 50 seconds or so).

"Solubility" product of Ag_2CrO_4. Approximate results were obtained by mixing standard solutions of $AgNO_3$ in 5 % gelatin and K_2CrO_4 in 5 % gelatin in equivalent quantities, allowing the gel to set and then causing $AgNO_3$, of different concentrations, to diffuse into the tubes, noting the tube which just gave faint precipitate at the surface (or, better, just a red coloration). Varying concentrations of entering $AgNO_3$ and of Ag_2CrO_4 in the gel all gave, for the same gelatin (5 % gel), a "solubility" product of the order of $(2{\cdot}2 \text{ to } 2{\cdot}9)10^{-7}$.

For 5 % ash-free gelatin the limiting "solubility" was between $N/166$ and $N/182$, giving a (mean) "solubility" product 1×10^{-7}. Bands of silver chromate can be obtained when concentrated $AgNO_3$ (*e.g.* N) diffuses into a gel containing Ag_2CrO_4 as weak as $N/1250$.

These results, rough as they are, indicate that the solubility product holds good for the "solubility" of Ag_2CrO_4 in gelatin.

The actual amount of Ag_2CrO_4 remaining in the clear spaces between bands must be less than the "solubility" measured as just described, *i.e.* lower than $0{\cdot}0085\ N$, because there must be subsequent separation of Ag_2CrO_4 due to the desaturating effect of that already separated. The reduction in solubility due to oncoming Ag, or to a less extent, to CrO_4, should not greatly affect the amount of total Ag_2CrO_4 actually found in the clear spaces, though it will precipitate some as solid particles in the clear spaces. This explains also why clear spaces formed in the early stages of diffusion subsequently fill (particularly noticed when rings are close together, as for example, when K_2CrO_4 is in the gel). The following are results showing the amount of Ag_2CrO_4 (calculated from the silver) in clear spaces—necessarily, to be wide enough for separate analyses, at some distance from the origin of the diffusion of the CrO_4 (or Cr_2O_7).

Table I.

(*a*) 0·1 *M* K_2CrO_4 diffusing into 0·01 *N* $AgNO_3$	(*b*) 0·2 *N* $K_2Cr_2O_7$ diffusing into 0·01 *N* $AgNO_3$
$[Ag_2CrO_4]$ in "clear" spaces	$[Ag_2CrO_4]$ in "clear" spaces
N	*N*
0·0076	0·0043
0·0068	0·0036
0·0061	0·0035
0·0061	0·0033
0·0046	0·0042

Obviously the concentration of the ion in the gel should not be very much greater than the limiting "solubility" if clearly spaced rings are required in the early stages of diffusion.

C. Development of the precipitate.

Once separation has occurred there is concentration of the precipitate by collection of the material from the intermediate zones. This is clearly seen if the formation of rings is followed by observation through the microscope, and particularly well if such observation is made when the rings are being produced under the influence of a superimposed E.M.F. An explanation of the mechanism of this concentration into more or less clearly defined bands is given by Jablczynski *et al.* [1923] who shows that it can be attributed to the growth of the relatively large particles at the expense of the smallest. This is a contributory effect in making bands more clearly defined; it is not the cause of band-formation. There is evidence in support of this theory in the behaviour of the primary rings which are often noticed when Ag_2CrO_4 forms in rings—particularly when $K_2Cr_2O_7$ is the internal reagent.

Where the main bands are rather close together, the primary bands show definite signs of being absorbed by the main bands. Where the main bands are relatively far apart, the central primary bands, being out of the "zone of attraction" into the main bands, may form a new "supplementary" band, and this probably explains the occurrence of bands in doublets and even triplets as frequently seen, in capillary tubes, where the rings are becoming more widely spaced, *i.e.* at some little distance from the surface where the electrolyte enters. Fig. 1, Plate V, showing the bands beyond the 18th band in a capillary tube, clearly suggests adsorption of the primary bands and indicates the formation of double bands.

D. Theory of formation of Liesegang rings.

(1) *Spacing of bands and its relation to the diffusion gradient of the entering salt (free ion).*

It has been found experimentally [Jablczynski, 1926] that the distances between bands are in the simple relationship

$$\frac{h_n}{h_{n-1}}=K, \quad \frac{h_n-h_{n-1}}{h_{n-1}-h_{n-2}}=K.$$

Morse [1930] states that "this indicates a ruling influence of diffusion and especially the influence of the diffusing (more concentrated) substance on ring formation and periodicity."

It is to be inferred that a constancy such as this depends on the cause(s) being of an unchanging nature. The diffusion of the entering ion fulfils this requirement; it proceeds so that the distance reached by the free ion is in accordance with $h/\sqrt{t}=K$. The corresponding requirement on the part of the ion in the gel is that its distribution, from the front of the last formed precipitate, throughout the unpenetrated part of the gel, shall remain reasonably constant. This is approximately true generally, but, as the ion contained in the gel moves forward to the entering ion, there must be some decrease in its concentration, with the consequence that the relation is not perfectly true over a long enough range, the change becoming noticeable in certain circumstances, such as when the concentration of the internal electrolyte is low and the entering ion has penetrated so far that its advance is very slow.

The effect on the spacing of the bands of variation in the concentration of the internal electrolyte is well illustrated by taking an exaggerated instance. For example, if a gradient of concentration of the internal electrolyte is produced by causing that electrolyte to diffuse into a gel, the other electrolyte which is subsequently caused to follow is thus diffusing into a gel containing electrolyte of gradually decreasing concentration (Fig. 2, Plate V); the second electrolyte may, instead, be introduced at the other end of the tube, thus travelling into electrolyte of increasing concentration.

A meaning can be found for the relationship as follows:

It is an experimental fact that the diffusion of the entering ion (or salt) X proceeds according to Fick's law whether or not any precipitating ion (salt) Y is contained in the gel. That is, although when precipitation does occur there is sudden separation of XY with temporary alteration of diffusing conditions, yet this does not amount to definite checking of the diffusion as the effect at any time is in accordance with the diffusion law (for a given external concentration C, $\frac{h}{\sqrt{t}}=K$). This is true so long as it is the "free" X which is considered.

In Fig. 3, CD represents the curve for "free" X when precipitation is just about to take place at A (h_{n-1}) where the concentration of X = metastable

"solubility," s. For a new precipitation to follow at B, (h_n), the concentration of "free" X at A, after precipitation at A, will have been made up to the original and have risen to an amount AF, so that CFE represents the "free" X curve when B is ready to precipitate, *i.e.* concentration at B has likewise reached the metastable "solubility," s.

Let it be assumed that when this value has been reached at B, the concentration of "free" X at A has reached a definite value, p, and that this is true for any ring when a new one is forming next to it.

For any given concentration of X along the tube, $\frac{h}{\sqrt{t}}=K$ for all positions of X of that given concentration: thus for concentrations of $X=s$, $\frac{h_{n-1}}{\sqrt{t_{n-1}}}=K_1$ and $\frac{h_n}{\sqrt{t_n}}=K_1$; for concentration $X=p$, $\frac{h_{n-1}}{\sqrt{t_n}}=K_2$.

$\frac{h_n}{h_{n-1}}$ then becomes $\frac{K_1}{K_2}=k=\left(\frac{\sqrt{t_n}}{\sqrt{t_{n-1}}}\right)$.

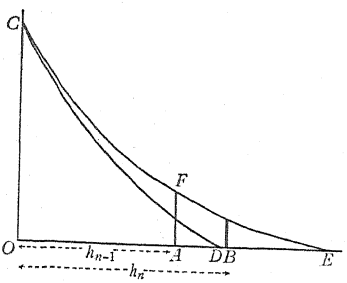


Fig. 3. CD and CE are curves for diffusion of the entering ion X.

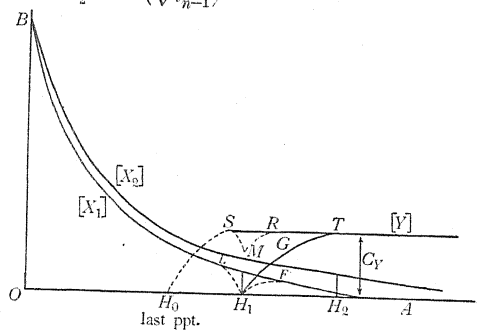


Fig. 4. Ordinate: Concentration (X and Y) Abscissa: Distance from surface.

So that, in order to satisfy the experimental facts that the ratio of distances of consecutive rings from the origin shall be constant, and that the times of formation shall be in accordance with the diffusion law, the requirements are:

(1) That ring-formation shall occur at a definite concentration of X (say $=s$). This follows from the fact that the concentration of Y, the other ion, is constant (uniformly distributed) so that, for the "solubility" product, which is constant, $[X]$ must reach a constant value.

(2) That when $[X]$ reaches this value (s), the concentration of "free" X at the ring previously formed must be of a certain constant value (p).

It is clear that the slope (or rather the flatness) of the diffusion curve governs the spacing of the bands, *e.g.*

M	K_2CrO_4 diffusing at 21° into 5 % gelatin, containing $N/100$ $AgNO_3$ gave	$h/\sqrt{t}=1{\cdot}085$
$M/10$	,, ,, ,, ,,	$=1{\cdot}20$
$M/50$	,, ,, ,, ,,	$=1{\cdot}48$

The effect on the spacing, of the concentration of the contained salt (ion), is likewise to cause decrease for higher concentration, so that the position of the point B is governed by the gradient of the concentration of the contained ion at the precipitate front and it must be such that B (H_2 in Fig. 4) is under the horizontal position of that curve, *i.e.* beyond the actual gradient of concentration of the contained ion. This becomes evident in the following theoretical visualisation of the mechanism of band-separation, based on diffusion and the conception of metastable solution as proposed by several other workers, *e.g.* particularly Fricke [1923], Morse [1930] and others. The importance attached

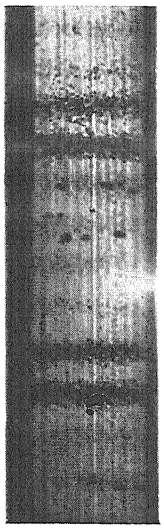
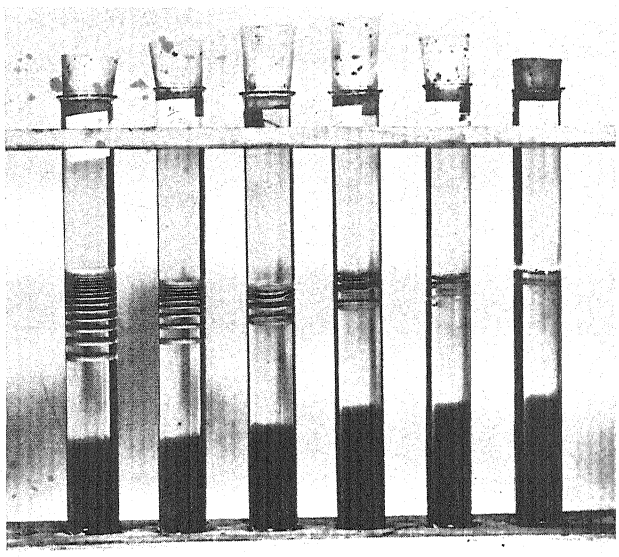


Fig. 1.

Fig. 2.



1 2 3 4 5 6

Fig. 7.

to diffusion gradients, as here emphasised, has been realised solely from the analytical work recorded and is considered to afford definite confirmation of the theoretical considerations described by Fricke.

Theory of formation of Liesegang rings.

For simplification of description reference is made only to the ions which form the precipitate, and they are considered to be of equal valency. X is used to represent the entering "free" ion, and Y the ion contained in the gel. Suitable conditions of gradient of concentration of "free" X and concentration of Y are presumed (Fig. 4).

C_X = external concentration of X.
C_Y = concentration of Y (as originally uniform in the tube).
S = "solubility" (metastable) product.
S_X = concentration of X required to give "solubility" product S with C_Y.

Concentrations are amounts above final solubility of the product XY. Consider precipitation occurring at a point H_1. Here the metastable "solubility" product will have been reached, and, as C_Y is constant (approximately), S_X for ion X must be constant when precipitation occurs. X and Y now precipitate at H_1 and consequently there is a drop in concentration of the free ions, to the zero line (final solubility) for X, and to $C_Y - S_X$ for Y, as shown by the dotted lines at H_1, LH_1F [Fricke, 1923] and SMR respectively. As the amount of X, at H_1 and onwards, builds up again, so the Y in excess at H_1 and its neighbourhood is precipitated and collected into the precipitate at H_1, with the consequence that the concentration of Y at H_1 is gradually falling and that of X increasing again.

Finally the concentration of Y at H_1 is down to the final solubility of XY (the ultimate solubility of XY being that in presence of excess of X) and the conditions when precipitation again occurs at H_2 are represented for Y by the gradient from C_Y at H_2 to zero at H_1 (H_1GT) and, for X, by the curve X_2. The changes then re-occur as described for H_1 (though the whole procedure must be considered in general as continuous, except for the actual separation of XY at concentration of $X = S_X$). The amount of Y remaining between one precipitate and the next is precipitated ultimately, so that, to obtain clear spaces, it is essential that C_Y should be relatively only little higher than S_X.

Position of H_2 (B of Fig. 3).

It will be noted that, in the diagram, the positions of H_1 and H_2 have been shown as under the horizontal portion of the concentration curve for Y. The reason of this is as follows:

Referring again to the precipitation at H_2, Y moves along the gradient C_Y to zero at H_1 until interrupted by precipitation at H_2 with the consequence that the average (distribution) concentration of Y (as precipitate) is greater to the left of the last ring than to the right of it (experimental evidence, p. 1093). The concentration of free Y at H_1 is finally "zero." When precipitation takes place at H_2, this movement of Y to H_1 ceases, and so no further Y goes to the left. Consequently, rings once completely formed, except for the absorption of Y remaining in the last space or two, will no longer increase in amount of precipitate (experimental evidence, Graph 12). If H_2 were at a point in the actual gradient of Y to C_Y then the average (distribution) of Y finally remaining as precipitate would, for that distance, be less than C_Y, instead of slightly greater, a condition which does not occur. Consequently the zones of precipitation are

such that Y is at its standard concentration. The following analytical results, Table II, *a*, *b*, from some of the results previously given as curves, also illustrate this. In each group the results represent analyses made of identical tubes of gel after diffusion had proceeded for increasing periods of time, *e.g.* 24, 120, 172, 216 and 312 hours, and show the concentration of the internal ion in the zone of the precipitate and just beyond it, *i.e.* before the last formed ring has much intensified by collection from its neighbourhood. Such figures cannot, of course, be exactly representative of the newness or otherwise of a band, or exactly of the concentration in a band not very sharply defined, but they serve as an indication. In the precipitate, results for $[Y]$ greater than C_Y indicate that the precipitate had been formed for some time.

Table II.

a

0·5 *N* $AgNO_3$, *X*, into 0·04 *N* K_2CrO_4, *Y*

Front edge of precipitate	Portion of clear gel adjacent to precipitate
$[CrO_4]$ *N*	
0·039	0·020
0·044	0·020
0·044	0·010
0·047	0·012
0·043	0·011
0·042	0·012

b

0·1 *M* $K_2Cr_2O_7$, *X*, into 0·0075 *N* $AgNO_3$, *Y*

Front edge of precipitate	Portion of clear gel adjacent to precipitate
[Ag] *N*	
0·0097	0·0069
0·0095	0·0072
0·0105	0·0054
0·0083	0·0059

In view of the results and conclusions so far given, it becomes a simple matter to decide what conditions are necessary for the formation of banded precipitates or for continuous precipitate, presuming suitably slight solubility of the precipitate and the concentration of the contained ion above the metastable "solubility" of the precipitate.

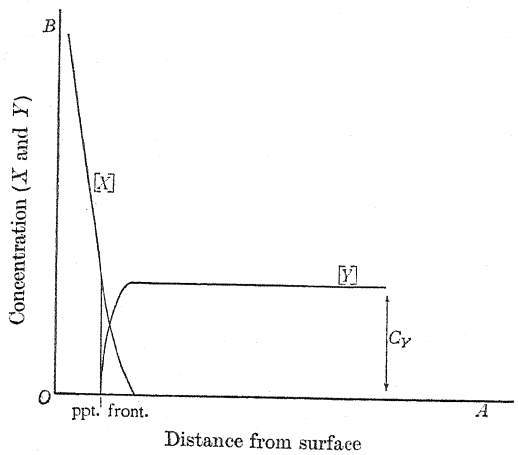


Fig. 5.

1. *Continuous precipitate* (Fig. 5).

The gradient of $[X]$ must be steep so that a slight movement along OA brings the requisite amount of X required for precipitation under C_Y. Likewise the gradient C_Y must be so steep that $[X]$, in an extremely small distance from

the precipitate front, comes to "solubility" concentration under the horizontal part of $[Y]$. This necessitates relatively high concentration of Y and much higher concentration of X. Unless C_X is really high compared with C_Y some band-formation will occur after diffusion has proceeded for some time and the gradient of $[X]$ has consequently become much less steep.

2. *Banded precipitate* (Fig. 6).

For the formation of widely spaced clearly separated bands, the gradient of $[Y]$ must be relatively flat, so that the next occurrence of the metastable limit, which will be for a position of the requisite amount of X under the horizontal portion of the $[Y]$ curve, will be some distance away from the last precipitate. The clearness of the spaces will depend on the amount of Y left between a new band and the previous one, and consequently the concentration of Y $[C_Y]$ should be low, this depending on the effect of X in reducing solubility of XY

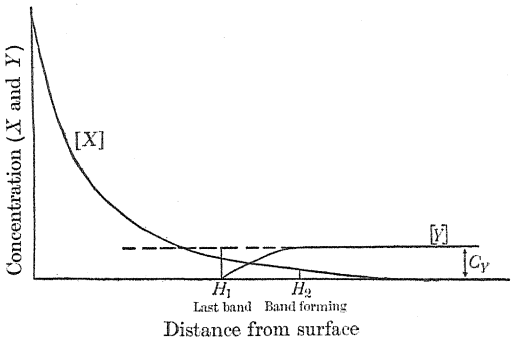


Fig. 6.

and also on the way in which XY can be absorbed into a band. The spacing will depend both on the concentration of Y, which must be low, and on the external concentration of X which also must be low (though, of course, higher than that of Y) in order that the gradient of $[X]$ shall be flat (as H_1 to H_2, Fig. 6).

As already explained, the spacing can be considered in terms of the diffusion of X only with allowance for the effect of $[C_Y]$ on it; *i.e.* as a balanced continuous process.

With conditions between those for continuous precipitate and for widely spaced clearly separated bands, any intermediate type of banding may be procured, *e.g.* rather close bands with fairly clear spaces; close bands with "spaces" filled with precipitate, the bands being only distinct as more intensely coloured and more compact; and so on.

When the gradient of $[X]$ is relatively steep and $[C_Y]$ is rather low, there will be a tendency for some continuous precipitate to form before the gradient of $[X]$ is flat enough to give banding. This is a matter of common observation.

If the $[X]$ gradient commences rather flat, it is possible that banding will commence at once. For example, Fig. 7, Plate V (1–6) shows the results of diffusion of $N/10$, $N/20$, $N/30$, $N/40$, $N/50$, $N/60$, K_2CrO_4 respectively into $N/100$ $AgNO_3$ in 5 % gelatin (for 64 hours).

Thus it is possible, allowing for conditions as described, to predict the character of band-formation and to adjust conditions accordingly.

REFERENCES.

Adair (1920). *Biochem. J.* **14**, 762.
Bolam and Mackenzie (1926). *Trans. Far. Soc.* **22**, 151, 162.
Bradford (1920). *Biochem. J.* **14**, 474.
Davies (1922). *J. Amer. Chem. Soc.* **44**, 2698.
Desai and Nabar (1931). *Nature*, **127**, 628.
—— —— (1932). *Trans. Far. Soc.* **28**, 449.
Fricke (1923). *Z. physikal. Chem.* **107**, 41.
—— and Suwelack (1926). *Z. physikal. Chem.* **124**, 359.
Hatschek (1922). *Biochem. J.* **16**, 475.
Hedges (1932). Liesegang rings and other periodic structures. (Chapman and Hall, London.)
—— and Henley (1928). *J. Chem. Soc.* 2714.
Hughes (1928). *J. Soc. Chem. Ind.* **47**, 1347.
Jablczynski (1926). *Kolloid Z.* **40**, 22.
—— and others (1923). *Bull. Soc. Chim.* **33**, 1392, 1592.
Loeb (1922). *J. Amer. Chem. Soc.* **44**, 213.
Morse (1930). *J. Physical Chem.* **34**, 1554.
—— and Pierce (1903). *Z. physikal. Chem.* **45**, 589.
Notboom (1923). *Kolloid Z.* **32**, 247.
Ostwald (1925). *Kolloid Z.* **36** (Suppl.), 380.
Steopoe (1924). *Bull. Chim. Soc. Romana Stiinte*, **27**, 51.
Stiles (1920). *Biochem. J.* **14**, 58.
—— (1921). *Biochem. J.* **15**, 629.
—— and Adair (1921). *Biochem. J.* **15**, 620.
Williams and Mackenzie (1920). *J. Chem. Soc.* **117**, 844.

CXLIX. STUDIES IN ZYMASIS.
VII. THE ESTIMATION OF THE ETHYL ALCOHOL AND ACETALDEHYDE CONTENT OF APPLES.

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THE following paper presents an account of the methods used in arriving at the alcohol numbers [Fidler, 1933, 1] given in the former papers of this series [Fidler, 1933, 1, 2; Thomas and Fidler, 1933].

Suggestive information has been gained by previous workers (see literature quoted by Kostytschew [1927] and Thomas [1925]) as to the progress of alcohol production by methods that had not been standardised. Little value, however, can be attached to numbers purporting to represent relations between alcohol and carbon dioxide production. Moreover, we consider that when anaerobic respiration forms one component of the total respiration it is essential to know the accuracy with which the various zymasic products and the respirable substrates have been measured, before any attempt is made to construct a balance sheet.

The results of this critical examination of a specialised analytical technique are presented in summary tables in this paper, and it will be seen that in all cases the standard deviations of the results are known. In view of the detailed discussion of the technique that was to be given in this paper, no mention of the accuracy of the methods was made in the earlier papers of this series, but only significant figures were given in the tables. It will now be seen that we are able to determine whether any experimental treatment can be said significantly to have increased the alcohol number of an apple.

The relation between the actual alcohol number of an apple at the end of an experimental treatment to the production of ethyl alcohol and acetaldehyde during the period of the experiment is discussed in appendices.

I. Estimation of ethyl alcohol and acetaldehyde in physical systems.

(The results of the different sub-sections are given in the summary Table I.)

A. IN AQUEOUS SOLUTIONS.

1. *Acetaldehyde.*

(*a*) *By the method of Ripper* [*1900*]. Ripper's method is so well known that the details need not be given here. It has been tested, using a pure specimen of acetaldehyde, purchased from Kahlbaum, and found to be accurate to within the limit given by Ripper, namely $\pm 0{\cdot}3$ %. In four trials of the method, using

different volumes of an acetaldehyde solution made up from a weighed quantity of the pure liquid, the correspondence between the weighed quantity of acetaldehyde and the quantity determined by the fixation with sodium hydrogen sulphite was within 0·1 mg. in quantities ranging from 19·7 mg. to 39·4 mg. of acetaldehyde.

It may be thought that an error would be introduced into the acetaldehyde figure found by this method, due to autoxidation of the sodium hydrogen sulphite. However, using $N/20$ iodine solution in the titrations, no oxidation of the bisulphite solution could be detected in an hour, the liquid standing with a little air in a corked flask in melting ice.

(*b*) *By oxidation with potassium dichromate.* It has been found that acetaldehyde may be determined by oxidation with potassium dichromate in acid solution, the resulting volatile acid being then distilled off and estimated by titration with standard NaOH solution.

A series of oxidations was carried out with amounts of acetaldehyde ranging from 0·8 to 78·7 mg. under standard conditions, the concentration of sulphuric acid being maintained at 2·5 % by volume and that of potassium dichromate at 5 % by weight.

Aqueous solutions of acetaldehyde of approximately the strengths required were standardised by Ripper's method, and 10 ml. were added to each of three bottles containing 10 g. pure potassium dichromate and 5 ml. pure sulphuric acid, dissolved in 185 ml. of distilled water. The bottles were closed by ground glass stoppers and allowed to stand 18 hours in melting ice, after which the contents were transferred to distilling flasks. The liquids were distilled down to about 50 ml., and water was then added to the contents of the flask. These distillations and additions of water were continued as long as any volatile acid distilled over. Each 500 ml. of distillate was titrated with $N/10$ CO_2-free NaOH, using phenolphthalein as an indicator, and the distillations were continued until 500 ml. of the distillate required an amount of NaOH equivalent to that required by a distillation of a mixture of 10 g. potassium dichromate, 5 ml. sulphuric acid and 200 ml. of water.

This last titration reading is not included in the calculation of the total acidity of the distillates. From the total amount of NaOH required, the acetaldehyde content of the original solution was calculated, on the basis of the equations:

$$2CH_3.CHO + O_2 \rightarrow 2CH_3.COOH,$$

and

$$CH_3.COOH + NaOH \rightarrow CH_3.COONa + H_2O$$

and in every case was found to be greater than that found by Ripper's method. Since we may rely on the accuracy of Ripper's method, it is clear that some acidic substance other than acetic acid is produced by the oxidation of acetaldehyde by potassium dichromate in acid solution[1].

Experiments carried out in triplicate in ten different concentrations within the range indicated above gave a mean percentage excess of acetaldehyde by the oxidation method, as compared with Ripper's method, of 10·1 %, with a standard deviation of 2·51. The results are plotted in Fig. 1, where a line has been drawn through the origin, making an angle of 47° 45′ with the x-axis. The

[1] Part of the distillate, carefully neutralised with CO_2-free NaOH, did not induce turbidity in baryta solution containing barium chloride, so that the excess acidity is not due to SO_2 or CO_2. It may be due to formic acid. Conant and Tongberg [1930] have shown that the normal course of the oxidation of acetaldehyde with ceric sulphate and sulphuric acid is to formic acid, and claim to have demonstrated α-oxidation of acetaldehyde in acid potassium dichromate solution.

tangent of this angle is 1·101. This means that the values of the abscissae exceed those of the ordinates by 10·1 %. This line passes through or very near to the points of the figure. The graph further shows that the deviation from the mean is of the order of 0·5 mg. in the lower concentrations, and never greater than 2·0 mg. in the high concentrations. The sections dealing with estimations of mixtures of ethyl alcohol and acetaldehyde will illustrate the value of the above oxidation method (*e.g.* see III A).

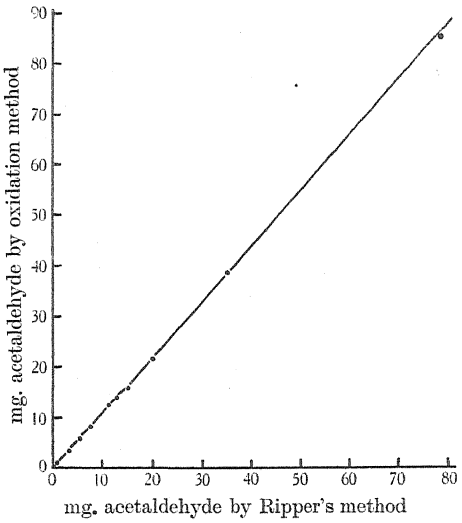

Fig. 1.

Further experiments, in which the concentration of the sulphuric acid was increased to 5 and 15 % and the time of oxidation to 3 days at 15°, gave figures which did not deviate from the mean value of 10·1 % excess by more than $2 \times$ S.D., so we may conclude that the conditions of the oxidation may be allowed to vary within limits. However, all oxidations of acetaldehyde described in this paper have been carried out in 2·5 % sulphuric acid for 18 hours at 0°.

2. *Ethyl alcohol.*

(*a*) *By oxidation with potassium dichromate.* Dox and Lamb [1916] describe a method[1] of estimating small quantities of ethyl alcohol by oxidation to acetic acid and titration of this compound with standard alkali. The method has been tested over a range of alcohol concentrations, and the results are given in Table I. The alcohol added was estimated by determination of the sp. gr. in a pyknometer, and reference to tables. All oxidations were carried out in 500 ml. solution, containing 12·5 ml. concentrated sulphuric acid and 10 g. potassium dichromate. The oxidation mixture was allowed to stand for half an hour at room temperature and then distilled, with repeated additions of water to the contents of the flask. The distillates were titrated against $N/10$ CO_2-free NaOH until the figure required to neutralise the distillate from the distillation of a mixture of sulphuric acid, potassium dichromate and water was reached. The first titration reading equal to this is included in the calculation of acidity.

[1] First described by Dupré [1867] and given in Allen [1901].

Table I. *Recovery of acetaldehyde and ethyl alcohol from physical systems.*

Substance added	Method of: Standardisation of solution	Method of: Estimation of recovery	No. of different weights of substances added	Total no. of observations	Range of weights of substances added (g.): From	Range of weights of substances added (g.): To	Mean percentage recovery	Deviations from mean percentage recovery: In lowest conc. +	In lowest conc. −	In highest conc. +	In highest conc. −	Maximum observed +	Maximum observed −	Standard deviation (S.D.)
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
(1) Estimation in aqueous solution:														
Acetaldehyde	Weighed amount of acetaldehyde in definite volume of water	Ripper	2	4	0·0197	0·0394	100·25	Nil	Nil	Nil	Nil	Nil	Nil	Nil
„	Ripper	Oxidation	10	10	0·0008	0·0787	110·1 (*a*)	3·0	—	—	1·0	3·0	6·0	2·51
Ethyl alcohol	Pyknometer	„	7	16	0·0421	0·2185	100·1	1·1	1·3	0·4	0·8	1·2	1·3	0·99
(2) Following steam-distillation of an aqueous solution:														
Acetaldehyde	Ripper	Ripper	6	15	0·00120	0·02126	98·0	5·2	1·2	—	0·9	5·2	3·1	1·97
„	„	Half distillate oxidised. Other half: Ripper	2	8	0·0292	0·0600	97·0 (*b*) 98·3	3·0 1·7	5·5 1·1	2·2 1·7	0·2 2·6	3·0 1·7	5·5 2·6	2·60 1·54
Ethyl alcohol	Pyknometer	Oxidation	7	17	0·0466	0·1718	100·2	1·4	2·3	—	1·5	1·4	2·3	1·09
Acetaldehyde	Ripper	Ripper	6	24	0·0058	0·0140	98·8	—	2·1	1·9	1·7	2·8	3·3	1·74
Ethyl alcohol	Pyknometer	Difference	2	24	0·0467	0·1167	100·4	3·0	1·8	0·4	1·3	3·0	1·8	1·03
Alcohol number	Sum	Oxidation	6+2 (*c*)	6+24	0·0525	0·1307	100·2	2·4	1·9	1·2	0·9	2·4	1·9	0·97

(*a*) Recovery calculated on assumption that all volatile acid is acetic acid.
(*b*) This figure cannot be compared with (*a*) since these recoveries were calculated by assuming that the acetaldehyde equivalent of the volatile acid was 100/110·1 (see Fig. 1).
(*c*) This series includes six experiments in which the recovery of acetaldehyde from the mixture was not estimated, but assumed to be 100 %.

Table I shows that the mean percentage recovery in sixteen trials of the above method, with amounts of ethyl alcohol ranging from 0·0421 to 0·2185 g., was 100·1, and the S.D. of the method was 0·99. This is a high order of accuracy for an organic analysis, and the method is ideally suited to the estimation of ethyl alcohol in low concentrations in aqueous solution.

B. In distillates obtained from steam-distillation of aqueous solutions.

It is pertinent to the present enquiry to know whether distillation in a current of steam and condensation of the distillates lead to any loss of ethyl alcohol, or acetaldehyde, from aqueous solutions containing these substances, either singly or in mixtures with each other. Details concerning the solutions which have been distilled are given in Table I. All solutions were distilled for 2 hours, the distillates being collected as described in Section III A.

1. *Acetaldehyde.*

It has been found impossible to avoid loss of acetaldehyde in steam-distillation, while taking the greatest care to guard against losses. When the acetaldehyde content of the distillate was determined by Ripper's method, the average recovery in fifteen trials, in which the weight of acetaldehyde ranged between 0·0012 and 0·0213 g., was 98 %, with a S.D. of 1·97. Using the oxidation method of estimation, the average recovery fell to 97 %, and the S.D. rose to 2·6. This error is of the same order as that introduced by the oxidation. So we conclude that, on the average, 3 % of this very reactive substance has been lost, possibly by undergoing some chemical change at the high temperature. It is possible for the loss to be as high as 8 % in extreme cases, although the maximum observed loss was 5·5 %.

2. *Ethyl alcohol.*

Seventeen trials of the effect of steam on ethyl alcohol solutions were carried out. In these the weights of ethyl alcohol varied from 0·0466 to 0·1718 g. The average percentage recovery was unaffected by the steam-distillation treatment, and the errors of estimation appeared to be of the same order as those recorded under A (2).

3. *Mixtures of ethyl alcohol and acetaldehyde.*

Mixtures of standard solutions of these substances in different proportions were distilled and the distillates worked up by the standard method described in Section III A. We now encounter for the first time in this discussion variations in the estimates of the "alcohol number." The standard method, it will be seen later, gives a direct measure of the acetaldehyde content and the alcohol number, but the ethyl alcohol concentration is given by the difference between these figures and should therefore be subject to a variation which is a function of the separate variances of the acetaldehyde and alcohol number figures. It is therefore all the more gratifying to find that the mean recovery of ethyl alcohol, as found by difference, was of the same order as that in the modified Dox and Lamb estimation (A (2)) and the standard deviations of the two sets of figures were essentially the same.

The figures in Table I provide evidence that the presence of ethyl alcohol does not affect the recovery of acetaldehyde in a steam-distillation.

II. Estimation of ethyl alcohol and acetaldehyde in biological systems.

Since it was uncertain whether ethyl alcohol and acetaldehyde could be recovered quantitatively from biological material by a steam-distillation technique, a series of experiments was carried out on the recovery of known quantities of these substances from sliced apple tissue.

The sliced tissue (York Imperial, Newton Wonder or Bramley's Seedling apples) was well washed and mixed, and to separate 100 g. samples were added definite volumes of standard solutions of ethyl alcohol or acetaldehyde. The results are given in summary in Table II, and are discussed in some detail in the subsections below. No appreciable differences could be detected between the basal ethyl alcohol or acetaldehyde contents of separate 100 g. samples of the mixed tissue, using the analytical methods described under III A. The tissue was freshly prepared for each series of experiments. Usually four tests were made of any given concentration of the substance studied. All the mixtures stood half an hour at room temperature in a closed distilling flask before the current of steam was started. A distillation time of 2 hours was taken as the standard, and the figures in column 9 of Table II indicate that such a period is a satisfactory one.

The knowledge obtained from the results of the experiments described in I B is very necessary, because at the present time it seems that steam-distillation appears to be the best method of recovering ethyl alcohol and acetaldehyde from plant tissues, and especially from apples, in a short enough space of time to preclude secondary changes.

(A) Acetaldehyde and ethyl alcohol supplied singly.

A comparison of the mean percentage recoveries of either of these two substances in purely physical systems and in the presence of apple tissue (Tables I and II) shows that there are no striking differences between the two sets of figures. (In the case of ethyl alcohol the two means are identical.) There appears, however, to be more swing about the mean in the figures obtained from the biological systems. Thus, in the case of the recovery of acetaldehyde, the estimated S.D. of the mean has risen from 1·97 to 2·44, and similarly for ethyl alcohol from 1·09 to 1·56. Thus the maximum deviation to be expected, as a pure matter of chance, once in every twenty estimations, has risen from 4 to 5 % for acetaldehyde, and from 2 to 3 % for ethyl alcohol.

(B) Mixtures of acetaldehyde and ethyl alcohol.

The alcohol number. In estimations of products of zymasis in fruits, where the size of the sample is so large that sampling errors can be neglected (a state of affairs rarely realised), the results of this subsection will be the most important.

We will consider at this stage only the estimates of the S.D. given in column 16 of Table II. These estimates have been calculated by the same formula as used in the earlier subsections and are comparable with those given in Table I. Although the mean recovery of either acetaldehyde or of ethyl alcohol is not altered by the presence of the apple tissue during the distillation, there is greater deviation from this mean value. Thus the estimated S.D. has risen in each instance. Our published results deal with the alcohol numbers of apples,

Table II. *Recovery of acetaldehyde and ethyl alcohol from biological systems.*

Substance added	Method of: Standardisation of solution	Method of: Estimation of recovery	Variety of apple	No. of different weights of substances added	Total no. of observations	Range of sum of weights of substance added + amount present in tissue (*a*): From	Range of sum of weights of substance added + amount present in tissue (*a*): To	Mean percentage recovery	Deviations from mean percentage recovery: In lowest conc. +	In lowest conc. −	In highest conc. +	In highest conc. −	Maximum observed +	Maximum observed −	Standard deviation (s.d.): Method 1 (*b*)	Standard deviation (s.d.): Method 2 (*c*)
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Acetaldehyde	Ripper	Ripper	York Imperial	4	4	0·0092	0·0305	95·2	4·8	—	0·8	—	4·8	4·3	3·75	—
			Newton Wonder	6	24	0·0025	0·0241	97·8	8·2	—	0·5	0·7	8·2	3·8	2·64	—
			Bramley's Seedling	6	24	0·0038	0·0254	98·1	3·5	—	—	0·5	3·5	2·0	1·62	—
			All three varieties	16	52	0·0025	0·0305	97·7	8·3	—	—	3·4	8·3	6·8	2·44	—
Ethyl alcohol	Pyknometer	Oxidation	York Imperial	6	6	0·0574	0·4257	99·4	0·6	—	—	1·0	1·4	1·0	0·68	—
			Newton Wonder	4	16	0·0258	0·1728	100·1	2·6	3·6	0·2	0·9	2·6	3·6	1·65	—
			Bramley's Seedling	5	20	0·0252	0·2396	100·6	1·3	—	2·7	7·5	2·7	7·5	1·95	—
			All three varieties	15	42	0·0252	0·4257	100·2	1·7	—	0·5	1·9	3·1	6·1	1·56	—
Acetaldehyde	Ripper	Ripper	Newton Wonder	6	24	0·0053	0·0212	99·6	0·4	5·3	2·9	—	3·8	3·0	2·12	1·77
			Bramley's Seedling	4	24	0·0054	0·0204	97·8	0·4	3·2	4·2	0·3	4·2	5·2	2·45	2·05
			Both varieties	10	48	0·0054	0·0212	98·7	1·3	4·4	2·0	0·8	4·7	4·4	2·28	1·92
Ethyl alcohol	Pyknometer	Difference	Newton Wonder	2	24	0·0255	0·0885	100	5·5	7·0	2·6	2·8	5·5	7·0	3·68	4·16
			Bramley's Seedling	2	24	0·0261	0·0986	100·5	4·9	5·5	2·7	4·5	4·9	5·5	2·86	3·16
			Both varieties	4	48	0·0255	0·0986	100·2	7·5	7·2	2·9	4·2	7·5	7·2	3·33	3·69
Alcohol number	Sum	Oxidation	Newton Wonder	6	24	0·0308	0·1097	99·7	6·1	—	0·3	2·2	6·1	3·4	2·38	1·53
			Bramley's Seedling	6	24	0·0315	0·1140	100	3·2	1·6	1·8	3·7	3·2	3·7	2·36	2·38
			Both varieties	12	48	0·0308	0·1140	99·8	6·0	—	2·0	3·5	6·0	3·6	2·33	2·00

(*a*) The basal values for acetaldehyde and ethyl alcohol contents of the tissues were determined by the method described in Section III. They are subject to errors of estimation, but it would be an unnecessary refinement to allow for these errors.

(*b*) Throughout Sections I and II, the actual ethyl alcohol and acetaldehyde contents of the tissues were known, and hence the recoveries of these substances could be expressed as percentage recoveries. Considering any separate series from either of these two Sections (*e.g.* the series of trials of the recovery of ethyl alcohol from Bramley's Seedling apple tissue) the s.d. is found by the formula $\text{s.d.} = \sqrt{\frac{\Sigma (x-\bar{x})^2}{n-1}}$ where $\Sigma (x-\bar{x})^2$ = the sum of the squares of the differences between each percentage recovery (x) and the mean percentage recovery of the series ($\bar{x}$), and n = the total number of experiments in the series. Thus $n-1$ gives the number of degrees of freedom. Only column 16 will be considered in this section.

(*c*) The method of calculation of column 17, and further reference to this column, is deferred until Section III, in the notes to Table III.

and, if perfectly even sampling is assumed, it will be seen from the S.D. of the alcohol number that a deviation of 4·66 % from the true value is to be expected once in every twenty estimations as a matter of chance.

Up to the present, our studies have not necessitated an accurate knowledge of the ethyl alcohol content of tissues (as distinct from the alcohol numbers), but were we to calculate these figures from the percentage figures for acetaldehyde and the alcohol number, we should expect the odds against any figure deviating by as much as 7 % from the true value to be 19 : 1 (again neglecting sampling error).

III. Variations between small samples.

The data given in the previous section are of use in demonstrating that the projected method is applicable to the assay of alcohol numbers of apples. The figures are of little use, however, in analysis of actual experimental results obtained with whole apples, since the errors introduced by random sampling greatly exceed the errors in the analytical technique. Estimates of total errors (which include sampling errors) for samples of two apples each have been obtained for two varieties of apples. The experiments described in Nos. IV, V and VI of this series [Fidler, 1933, 1, 2; Thomas and Fidler, 1933] were done with samples ranging from one to three mature fruits, for reasons given in the sixth paper. Thus the samples for the experiments described in this section were of two apples each. As will be seen later, it is easy to calculate the error for a sample of any other size.

The results are given in Table III, and are discussed in Subsection B. Acetaldehyde and ethyl alcohol were formed in the apples by exposing them to the experimental conditions summarised in Table III, in the apparatus described by Fidler [1933, 1], for definite lengths of time. The experiments were arranged in groups, each of which comprised three comparable samples of two apples each. At the end of the experimental treatment, the separate samples of each group were distilled and the acetaldehyde and ethyl alcohol contents of the distillates determined in the manner described in Subsection A.

The figures given in Table III for ethyl alcohol and the alcohol number include the transpired alcohol, as did the figures in the earlier papers. This transpired alcohol is determined as described in Appendix II.

The apples were from the population stored for our use by Dr Cyril West, at the Ditton Laboratory, East Malling, Kent, during the season 1932–3.

A. Apparatus and method.

After removal of the apples from the experimental conditions, the alcohol and acetaldehyde were extracted by steam-distillation in the apparatus first used and described by Thomas [1925].

Passing through the stopper of a 2-litre flask, containing 1500 ml. water, is the narrow stem of a glass cylinder, 30 in. by 2½ in., the top of which is in communication by means of a splash trap with a 10 in. water condenser of the double surface type. The cylinder is well lagged with cotton-wool. The condenser is in turn connected to a tube passing through the stopper of a 1-litre flask to within ¼ in. of the bottom. This flask is connected to a second similar receiver. Each receiver contains 50 ml. distilled water and stands in a trough filled with melting ice.

Before use, the apparatus is disconnected at the top of the glass cylinder, and the water in the flask is boiled until the issuing steam has heated the walls of the cylinder. The apples are now cut, each into eight pieces, and dropped into the cylinder, where they are retained by a circle of

perforated zinc, the edges of which are bound with rubber. The apparatus is immediately reconnected and distillation proceeds for 2 hours.

When the distillation is completed, the distillates are combined and made up to 1 litre with water. Half of this liquor is used for the determination of acetaldehyde by Ripper's method[1] and the other half is oxidised by the addition of a solution of 15 ml. concentrated sulphuric acid and 25 g. potassium dichromate in 85 ml. water. The oxidation is allowed to proceed for 18 hours in ice. The conditions may be allowed to vary a little, as indicated in Section I A 1 (*a*), which also describes the method of distillation of the oxidation-liquors. It is advantageous to use Claisen flasks for these last distillations, and it is important that no traces of colour should be present in the acid distillates. The liquid in the flasks should be distilled to small bulk (about 50–60 ml.) after each addition of water. In this way it is found that three additions and evaporations of water will suffice to carry over very large amounts of volatile acid.

The calculations of the ethyl alcohol content, and the alcohol number are carried out in the following manner.

Let A = ethyl alcohol content of the distillate.

Let B = acetaldehyde content of the distillate (by Ripper's method).

Then the ethyl alcohol equivalent of $B = \frac{44}{46} B$.

On oxidation, B will yield volatile acid equivalent to $\frac{11^*}{10} \times \frac{44}{46} B$, expressed as ethyl alcohol.

C, the total ethyl alcohol equivalent of the distillate from the oxidation

$$= A + \left(\frac{11}{10} \times \frac{44}{46} B\right) \text{ or, } A = C - 1{\cdot}05\, B.$$

The alcohol number $= A + \frac{44}{46} B = A + 0{\cdot}96\, B$.

Of the two alternative methods of approach to the problem of estimation of ethyl alcohol in the presence of acetaldehyde, namely (1) removal of the acetaldehyde as the initial step in the analysis, and (2) oxidation of the whole solution after estimation of the acetaldehyde fraction by Ripper's method, the second has not previously been used. Despite numerous criticisms, it has been the universal practice of workers studying the course of fermentation to follow the first procedure. The acetaldehyde has been removed by, *e.g.* sodium hydrogen sulphite [Kostytschew and Hübbenet, 1912], or *m*-phenylenediamine hydrochloride [Neuberg and Hirsch, 1919]. The solutions are boiled under a reflux condenser to ensure condensation of the reagent with acetaldehyde, neutralised and evaporated at reduced pressure to remove the ethyl alcohol, which is condensed, or collected in sulphuric acid [Thomas, 1925]. The latter is probably the better practice.

Tomoda [1929] reviewing these, and related methods, describes them as "tedious and troublesome in manipulation" and states that loss of ethyl alcohol or incomplete removal of acetaldehyde may readily occur.

[1] *Estimations of acetaldehyde in the presence of HCN and H_2S.* As yet unpublished work by Thomas in 1929–31 and Thomas and Fidler during 1931–3 on the effect of HCN and H_2S on zymasis by apples necessitated slight modifications of Ripper's method. The blue end-point with starch and iodine is impermanent in the presence of HCN, and practice is needed in order to judge the correct end-point. With care, however, this may readily be done. The question is more involved, however, in the presence of H_2S, for iodine is reduced by H_2S. Our procedure when the distillates have contained this substance has been to oxidise one-third only, using one-third for the estimation of H_2S by titration with iodine. An amount of $N/20$ iodine equivalent to that used in this titration was then added to the third portion of the distillate, oxidising the H_2S. H_2S thus being removed, the estimation of acetaldehyde proceeded in the normal manner.

* From Fig. 1.

Table III. *Errors in estimations with samples of two apples each.*

Variety of apple	No. of exp. (in order of conc. of ethyl alc.)	Date exp. begins	Initial weight of sample (g.)	% oxygen in atmosphere of exp.	State of apples	Duration of exp. (hrs.)	g. per 100 g. apple per 100 hrs.: Acetaldehyde	g. per 100 g. apple per 100 hrs.: Ethyl alc. (allowing for transpired alc.)	% alc. no.	Increase in % alc. no. in 100 hrs. exposure to gas	% CO_2 production (g./100 hrs.)	CO_2 : alcohol no. (both expressed as gram-molecule equivalents)	S.D. (*a*): Acetaldehyde %	S.D. (*a*): Alc. no. or ethyl alc. %	S.D. (*a*): CO_2 %	S.D. (*a*): CO_2 : alc. no. %	2 √2 (S.D.) (*b*): Acetaldehyde %	2 √2 (S.D.) (*b*): Alc. no. or ethyl alc. %	2 √2 (S.D.) (*b*): CO_2 %	2 √2 (S.D.) (*b*): CO_2 : alc. no. %
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Newton Wonder	1 *a*	17. xi. 32	275·0	7·0	Green	96·0	0·0003	0·0117	0·0120	—	0·2389	—								
	b	,,	290·0	,,	,,	,,	0·0003	0·0091	0·0094	—	0·2461	—								
	c	,,	279·0	,,	,,	,,	0·0003	0·0117	0·0120	—	0·2475	—								
	2 *a*	26. x. 32	225·0	5·0	Green	72·0	0·0011	0·0137	0·0148	—	0·2315	—								
	b	,,	232·0	,,	,,	,,	0·0011	0·0138	0·0149	—	0·2169	—								
	c	,,	235·0	,,	,,	,,	0·0008	0·0134	0·0142	—	0·2137	—								
	3 *a*	19. x. 32	260·0	Nil	Green	119·0	0·0013	0·1521	0·1534	0·1500	0·2538	100:56·5								
	b	,,	306·0	,,	,,	,,	0·0014	0·1764	0·1778	0·1778	0·2760	100:60·4	13·9	8·7	5·0	6·2	40	25	14	18
	c	,,	296·0	,,	,,	,,	0·0017	0·2009	0·2026	0·1992	0·3160	100:65·9								
	4 *a*	7. ii. 33	222·5	3·5	Yellow	71·5	0·0027	0·2177	0·2204	—	0·3077	—								
	b	,,	213·0	,,	First sign of	,,	0·0035	0·2290	0·2325	—	0·3164	—								
	c	,,	217·5	,,	wrinkling	,,	0·0027	0·2210	0·2237	—	0·3080	—								
	5 *a*	10. xi. 32	226·5	Nil	Green	95·5	0·0042	0·2442	0·2485	0·2430	0·3430	100:67·8								
	b	,,	226·0	,,	,,	,,	0·0036	0·2455	0·2485	0·2428	0·3153	100:73·6								
	c	,,	235·0	,,	,,	,,	0·0033	0·2418	0·2451	0·2394	0·3294	100:69·5								
Bramley's Seedling	6 *a*	16. xii. 32	256·0	21·0	Green to	96·0	0·0030	0·0611	0·0641	—	0·4033	—								
	b	,,	240·5	,,	yellow-green	,,	0·0026	0·0566	0·0592	—	0·3922	—								
	c	,,	256·0	,,	,,	,,	0·0026	0·0620	0·0645	—	0·3748	—								
	7 *a*	1. xii. 32	261·5	7·6 then in	Green	48·5 + 240	0·0047	0·0998	0·1045	—	0·3280	—								
	b	,,	281·5	air 10 days	,,	,,	0·0045	0·0920	0·0965	—	0·2638	—								
	c	,,	250·0	at 15°	,,	,,	0·0046	0·0964	0·1010	—	0·2927	—								
	8 *a*	1. ii. 33	223·5	3·5	Yellow-green	95·0	0·0031	0·1376	0·1407	—	0·2828	—								
	b	,,	228·5	,,	,,	,,	0·0027	0·1151	0·1178	—	0·2444	—	8·9	6·3	7·5	4·4	25	18	21	12
	c	,,	222·5	,,	,,	,,	0·0026	0·1234	0·1260	—	0·2601	—								
	9 *a*	24. xi. 32	260·5	Nil	Green	95·5	0·0040	0·3633	0·3673	0·3632	0·4085	100:85·0								
	b	,,	265·0	,,	,,	,,	0·0030	0·3269	0·3299	0·3258	0·3625	100:86·0								
	c	,,	262·5	,,	,,	,,	0·0034	0·3649	0·3683	0·3642	0·3958	100:92·1								
												Considering both varieties together ...	12·1	7·7	6·2	5·6	34	22	18	16

(*a*) *The* S.D. In the experiments described in this section where the variation between samples of two apples, considered in groups of three samples, is studied, the actual contents of acetaldehyde and ethyl alcohol were unknown. Thus deviations can only be expressed, within each group, as deviations from the mean value for the group. The formula used in Sections I and II (see note (*b*) to Table II) for estimating the S.D. cannot be used in this case. We have instead to determine the square of the difference between each individual value within a group and the mean value of the group, and to sum the resulting figures for the whole series. We thus obtain an expression: $\Sigma d^2 = \Sigma (x-\bar{x}_1)^2 + \Sigma (x-\bar{x}_2)^2 \ldots + \Sigma (x-\bar{x}_m)^2$, where x is any individual value and $\bar{x}_1$, $\bar{x}_2$, and $\bar{x}_m$ are the different mean values for the different groups. The S.D. is given by $\sqrt{\frac{\Sigma d^2}{n-m}}$ where n = total number of experiments, m = total number of groups of experiments, and $n-m$ = number of degrees of freedom.

It is useful to know how far any result may differ from the mean value of the population, and 2 (S.D.) will give the percentage deviation from the mean within which 95 % of the observations will lie, as a matter of chance.

(*b*) It is often useful in biological work to know the probability of two independent observations differing by a given amount. The odds are 19 to 1 against any two random samples from the same population of apples differing by more than the percentage values given in this column. The well-known expression for this is 2 $\sqrt{2}$ (S.D.).

(*c*) All experiments were carried out at 23°.

The alternative procedure of oxidising the whole solution has the merit of being simple in manipulation, involving only those operations which would be necessary in estimating the alcohol content of a solution were it first of all freed from acetaldehyde. Little apparatus is necessary and, since the oxidation proceeds overnight, no time is lost.

B. Results.

The results of the experiments on Newton Wonder and Bramley's Seedling apples are given in Table III.

When the estimates of S.D. given in Table III are compared with those given in column 17 of Table II, it is immediately evident that random sampling has introduced an error greatly in excess of that due to the actual estimations.

From Table III it is seen that there must be very great differences between the values for any zymasic product in different samples of two apples each, before it can be said with certainty that the values are significantly different. Thus *e.g.* the alcohol numbers of two samples, each of two Bramley's Seedling apples, from different experimental conditions, must differ by more than 23 % before we are certain that the different conditions of the experiments have resulted in different values for the alcohol numbers.

Fortunately, there exists a well-known method for reducing the error introduced by random sampling. If the S.D. of a single observation is known, then the S.D. of the mean of n observations from comparable samples is $\frac{\text{S.D.}}{\sqrt{n}}$. This formula may be used in another way. Thus the S.D. of a sample of N apples, when the S.D. of a sample of size n is known is given by $\text{S.D.}_N = \frac{\text{S.D.}_n}{\sqrt{N/n}}$. If *e.g.* the sample size is increased to 20 apples, the S.D. of a single observation will be reduced to a value which is $\frac{1}{\sqrt{10}}$ times that of an observation from a sample of two apples.

The values for columns 14 to 21 of Table III for a sample of N apples may be found by multiplying the values given in the table by $\frac{1}{\sqrt{N/2}}$. It will be seen that the S.D. of an observation from a sample of 20 apples is relatively small. If the standard size of the sample were increased to such a great extent, it would, of course, be desirable to redetermine by experiment the value of the S.D.

Summary.

1. Commencing with the estimation of acetaldehyde and ethyl alcohol in purely physical systems, and then passing to biological systems, a technique has been evolved which is applicable to the assay of these substances in apples.

2. The standard deviations of the method described have been determined.

3. The method finally adopted is fairly rapid, and its accuracy is sufficient for the work reported in previous papers.

4. In brief, the method consists of a preliminary steam-distillation for 2 hours to rid the fruit of ethyl alcohol and acetaldehyde. Half the distillate is then oxidised to acetic acid, giving a measure of the alcohol number, while the acetaldehyde in the other half is estimated by Ripper's method. The ethyl alcohol figure is found by subtraction.

5. Apples should be distilled as soon as possible after removal from experimental conditions, as otherwise losses of zymasic products may occur (see Appendix I).

In addition to the acknowledgments to Mr Meirion Thomas, the Department of Scientific and Industrial Research, and to Armstrong College, which have been made previously [Fidler, 1933, 1], I wish to record my indebtedness to Mr J. Wishart, Reader in Statistics in the University of Cambridge, for his helpful criticisms of the methods of calculation of the standard errors.

Appendices.

THE RELATION BETWEEN THE PRODUCTION OF ACETALDEHYDE AND ETHYL ALCOHOL DURING THE COURSE OF THE EXPERIMENT AND THE AMOUNTS FOUND IN THE FRUIT AT THE TIME OF ANALYSIS.

The alcohol number of apples at the end of an experiment, determined by analysis, is not a true measure of the production of ethyl alcohol and acetaldehyde during the course of the experiment, especially when the analysis is deferred until some time after the fruit has been removed from the experimental conditions.

It has been shown that acetaldehyde disappears from apple tissue on exposure to air[1]. The results of experiments demonstrating this are given in Appendix I.

Ethyl alcohol is transpired by apples, but the loss from the fruit may be measured. The method of estimation and the magnitude of the escape are given in Appendix II.

APPENDIX I.

The loss of acetaldehyde from apples exposed to air.

The work described in this appendix was carried out solely with a view to determining how long analyses of apples could be delayed at the end of an experiment without affecting the acetaldehyde content.

The results may later be useful in considering the metabolism of acetaldehyde[2].

1. *Method.*

In many cases, a definite amount of acetaldehyde solution (standardised by Ripper's method) was added to a known weight of sliced apple tissue, the acetaldehyde content of which had been determined. In the majority of the experiments, the acetaldehyde was formed in the apples by exposing them to nitrogen or carbon dioxide-oxygen mixtures as described by Thomas [1925]. Analyses were carried out on samples immediately on re-exposure to air, and after different periods of aeration. All analyses of fruit were carried out by steam-distillation followed by acetaldehyde estimation by Ripper's method.

2. *Results.*

The results of the investigation are given in Table IV.

It is to be concluded from Table IV that loss of acetaldehyde may occur from apples exposed to air, and that this loss may be serious if the aeration is prolonged. Therefore analyses of apples for acetaldehyde should be carried out as soon as possible after removal from experimental conditions.

[1] Experiments by Thomas [1925] indicated that there was no loss of ethyl alcohol after several days' exposure to air, but work now being carried out seems to suggest that disappearance of ethyl alcohol may take place in special circumstances.

[2] The oxidation of acetaldehyde is one of the aspects of the current researches in this series. It is for this reason that a discussion of the cause of the acetaldehyde loss is not entered upon here.

Table IV. *The change in concentration of acetaldehyde in apple tissue.*

Variety of apple	State of apple	Condition of exp.	No. of hours during which mixture of acetaldehyde and apple tissue was exposed to air	Amount of acetaldehyde present in system (g.)		Percentage loss of acetaldehyde	Whether or not the loss is a significant one
				At beginning of exp.	At end of exp.		
York Imperial	—	Acetaldehyde added in soln.	0·5	0·0092	0·0093	Nil	—
,,	—	,,	0·5	0·0305	0·0277	9·1	Yes
,,	—	,,	0·5	0·0183	0·0175	4·5	No
,,	—	,,	0·5	0·0124	0·0117	5·6	No
,,	Senescent	,,	24	0·0266	0·0205	23·0	Yes
,,	,,	,,	44	0·0325	0·0166	49·0	Yes
Newton Wonder	Young	,,	48	0·0362	0·0334	8·5	Yes
,,	,,	Acetaldehyde formed in N_2	72	0·0033	Nil	100·0	Yes
,,	4 months in store	Acetaldehyde formed in 70 % CO_2-30 % O_2	97	0·0034	0·0001	97·0	Yes
,,	,,	,,	97	0·0046	0·0012	74·0	Yes
,,	5 months in store	,,	72	0·0104	0·0085	18·2	No
,,	,,	,,	72	0·0225	0·0136	39·1	No
,,	7 months in store	,,	48	0·0275	0·0111	59·6	Yes
Winesap	—	,,	72	0·0140	0·0106	24·3	No
Lord Suffield	Very young	,,	73	0·0320	0·0147	54·0	Yes
,,	,,	,,	101	0·0097	0·0034	64·9	Yes

Appendix II.

Transpiration losses.

A. *Ethyl alcohol.* Earlier workers on the subject of carbon dioxide : alcohol ratios realised that the experimental material may lose ethyl alcohol by transpiration. Kostytschew in his text-book [1927] cites the use of an ice-cooled wash-bottle containing distilled water to trap ethyl alcohol. In the researches already described in this series, concentrated sulphuric acid has been used successfully for this purpose.

1. *Method.*

As described in an earlier paper [Fidler, 1933, 1], the gas stream issuing from the fruit chamber passed through a wash-bottle containing 50 ml. of concentrated sulphuric acid. This treatment was shown to rid the gas of all appreciable amounts of ethyl alcohol, the method being checked as follows. A solution of ethyl alcohol in water was standardised by the oxidation method, and definite volumes were measured out into wash-bottles. Air was bubbled through each of these bottles at about 3 litres per hour, for definite time periods at room temperature (12–15°). The issuing air passed through each of two wash-bottles containing 50 ml. of concentrated sulphuric acid. The results are given in Table V.

Table V shows that all the ethyl alcohol escaping from the solution is trapped in the first wash-bottle containing sulphuric acid. The original Dox and Lamb [1916] aeration method of estimating ethyl alcohol made use of the formation of the non-volatile ethyl hydrogen sulphate in this manner.

Table V. *Trapping of ethyl alcohol in sulphuric acid.*

Amount of ethyl alcohol in wash-bottle (g.)		Loss of ethyl alcohol from wash-bottle (g.)	Period (days)	Ethyl alcohol trapped in sulphuric acid (g.)		Net loss or gain of ethyl alcohol (g.)
Beginning of aeration	End of aeration			First wash-bottle	Second wash-bottle	
0·1880	0·1022	0·0858	3	0·0853	Nil	−0·0005
0·1880	0·1290	0·0590	3	0·0605	,,	+0·0015
0·1880	0·1650	0·0230	1	0·0230	,,	Nil
0·1880	0·1650	0·0230	1	0·0248	,,	+0·0018
0·1880	0·0924	0·0956	5	0·0943	,,	−0·0013
0·1880	0·0819	0·1061	5	0·1055	,,	−0·0006

2. *The ratio alcohol content : alcohol escape.*

There is no need to give a detailed account of the magnitude of the escape of ethyl alcohol from apples, but a few examples will be of interest in indicating the values we have observed.

In our experiments on Newton Wonder and Bramley's Seedling apples at 23° during 1930–33, the mean value for the transpired alcohol was 8 % of that contained in the apple. During November and December 1933, an experiment was carried out on Sturmer Pippins in nitrogen at 20°. When the ethyl alcohol content of the tissues had risen to 0·2 % (after 12 days) the escape totalled 0·006 %, but when the alcohol content reached a value of 0·6 % (after 45 days) the escape totalled 0·076 %, or 15 % of the content. This figure has been exceeded with Bramley's Seedling apples.

Thus large errors may be introduced if no account is taken of ethyl alcohol loss by transpiration.

B. *Acetaldehyde.* We have, as yet, few figures for acetaldehyde escape, but such as we have indicate that there is little probability that appreciable error would be introduced into the alcohol figures by this means.

REFERENCES.

Allen (1901). Commercial organic analysis, **1**, 102. (Churchill.)
Conant and Tongberg (1930). *J. Biol. Chem.* **88**, 701.
Dox and Lamb (1916). *J. Amer. Chem. Soc.* **38**, 2561.
Dupré (1867). *J. Chem. Soc.* **20**, 495.
Fidler (1933, 1). *Biochem. J.* **27**, 1614.
—— (1933, 2). *Biochem. J.* **27**, 1622.
Kostytschew (1927). Plant respiration. (P. Blakiston's Sons, Philadelphia.)
—— and Hübbenet (1912). *Z. physiol. Chem.* **79**, 363.
Neuberg and Hirsch (1919). *Biochem. Z.* **98**, 141.
Ripper (1900). *Monatsh. Chem.* **21**, 1079.
Thomas (1925). *Biochem. J.* **19**, 927.
—— and Fidler (1933). *Biochem. J.* **27**, 1629.
Tomoda (1929). *J. Soc. Chem. Ind.* **48**, 77 T.

CL. THE EFFECT OF THE OXIDATION-REDUCTION POTENTIAL OF THE MEDIUM ON THE GROWTH OF TISSUE CULTURES.

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THE peculiarly high aerobic and anaerobic glycolysis of malignant cells suggests that they may be able to survive conditions of anoxaemia which are fatal to normal tissue cells. Warburg *et al.* [1924] have suggested the possibility of a connection between the increased power of glycolysis shown by tumour cells and their irregular and unrestrained growth. It becomes therefore of some importance to know how normal and malignant cells behave when given a poor oxygen supply while growing *in vitro*. The point has been studied by several workers, but their results have been conflicting.

Wind [1926] found that the Rous chicken sarcoma could grow under strictly anaerobic conditions which inhibited the growth of 10-day chick embryo heart tissue. Wright [1928] also found that malignant cells grew and divided at lower oxygen pressures than that in which normal embryonic tissue could grow; but he did not find that malignant cells would grow under strictly anaerobic conditions. He gives the lowest partial pressure of oxygen at which mitosis occurred in each tissue he examined, as follows:

	mm. O_2
Embryo chick heart	12
Sarcoma (J.R.S.)	6
Carcinoma (M 2164)	3

Fischer [1928] came to the opposite conclusion and reports that malignant cells survived less well under low pressures of oxygen when compared with normal cells.

While our experiments were in progress Laser [1933] published a paper in which he stated that if a culture of normal embryonic tissue were first allowed to grow for 24 hours in a normal atmosphere, it would then continue to grow well for several days under strictly anaerobic conditions. One or two experiments made to test this, reported later in this paper, do not support his conclusions.

It occurred to us that possibly the growth and division of tissue cells in such experiments might be governed more directly by the oxidation-reduction potential of the culture medium than by the concentration of oxygen in the gas phase. That the oxidation-reduction potential of a culture medium might have a real biological significance is suggested by the studies of Knight and Fildes [1930]. They found that germination of the spores of tetanus bacilli occurs only on the negative side of a certain very definite oxidation-reduction potential of the culture medium ($E_h = 110$ mv., when the p_H of the medium $= 7{\cdot}6$).

The E_h of the culture medium they employed fell spontaneously when a stream of purified nitrogen was bubbled through it. They were able to maintain the E_h at any required level by mixing with the purified nitrogen varying proportions of unpurified nitrogen, containing traces of oxygen. The partial pressure of oxygen which would be theoretically in reversible equilibrium with such a solution, $E_h = +110$ mv., would be about 10^{-44} atmospheres [Clark, 1928; Michaelis, 1930]. It is obvious therefore that the oxygen used in these experiments was not in reversible equilibrium with the oxidation-reduction systems of the medium. The definite and consistent results obtained by Knight and Fildes suggest that the germination of tetanus spores took place when a certain level of E_h was reached by the oxidation-reduction systems in the medium, rather than when a particular low concentration of oxygen was present. It is possible that at low oxygen pressures tissue cells also are affected more by the E_h of the culture medium in which they grow than by the oxygen pressure of the gas or of the solution. We have indeed no direct evidence that this is so; the possibility stimulated the performance of this series of experiments.

The purpose of this paper is to record how the growth and division of cells from the embryo chick heart may be correlated with the E_h of the medium in which it is suspended. In a later paper we hope to present a similar correlation between the growth of tumour cells and the E_h of the culture medium.

Experimental.

Preparation of medium and cultures.

In the earlier experiments the salt solution used as the basis of the medium was the phosphate solution recommended by Pannett and Compton [1924]. This fluid was chosen because if Tyrode solution containing bicarbonate were employed, then on passing a stream of nitrogen or air through the medium a considerable quantity of CO_2 would be removed, and the p_H would move too much towards the alkaline side. Media containing the correct concentration of Ca^{++} cannot however be adequately buffered by phosphate, on account of the precipitation of calcium phosphate. The addition of serum also complicates matters. In the later experiments, therefore, the usual Tyrode solution was used, and special gas mixtures containing 2·5 % carbon dioxide were employed. This amount of carbon dioxide kept the p_H of the medium at about 7·4, and in this respect much better control of the conditions was obtained. This change in the nature of the medium did not produce any apparent change in the behaviour of the cultures during an experiment.

Serum was obtained by allowing blood taken from the wing vein of a fowl to clot and the clot to contract and express the serum at 38°. The serum was centrifuged before use. Embryo extract was prepared from a fine mince of two 11-day or three 10-day chick embryos by the addition of 10 ml. Tyrode solution followed by centrifuging. The tissue used was taken from the apex of the heart of a 10–11-day chick embryo. Small fragments were explanted on to $\frac{3}{4}$ in. coverslips in a thin film of a mixture of equal parts of fowl plasma and embryo extract, and this mixture was allowed to clot. 15 ml. of a solution made up of 25 % embryo extract, 10 % serum and 65 % Tyrode solution were placed in a small beaker 6·5 cm. high and with a diameter of 3·5 cm. Three of the coverslip cultures were then placed in the beaker, standing on their edges on the bottom and leaning against the sides in an almost vertical position. The beaker was then closed by the rubber bung carrying the electrodes, gas

inlet and outlet tubes and agar bridge. The whole of the above process was carried out with sterile fluids and apparatus and under strictly aseptic conditions. The bung with its appurtenances was autoclaved while supported in the mouth of a boiling-tube half-filled with distilled water. Just before placing the bung in the culture vessel the tip of the tapered end of the agar bridge, which had been sealed, was broken off so that the KCl-agar might make contact with the medium in the beaker. The outer ends of the gas inlet and outlet tubes were tightly plugged with cotton wool (Fig. 1).

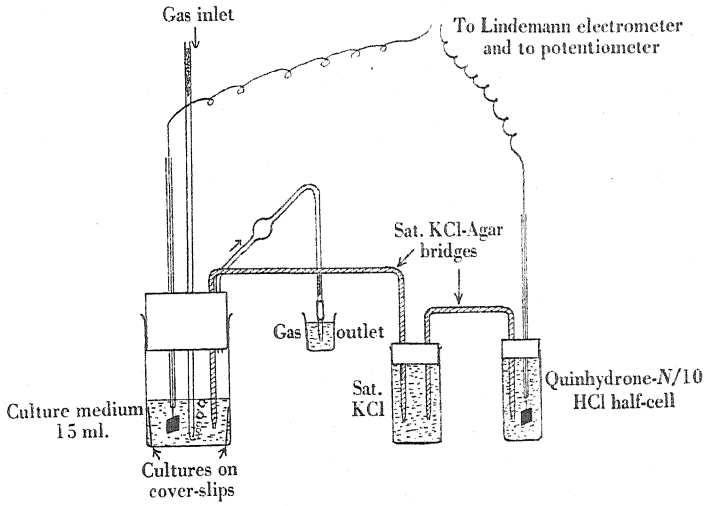


Fig. 1.

Preparation of the gas stream.

Nitrogen containing 2·5 % carbon dioxide was obtained from the British Oxygen Co. This mixture contained about 0·2 % of oxygen, which was removed by passing the gas through two sintered glass wash-bottles containing a solution of chromous chloride. This has the advantage of absorbing the last traces of oxygen without affecting the carbon dioxide [Warburg and Christian, 1931]. Unpurified nitrogen and 2·4 % carbon dioxide from another cylinder were added to the stream of purified gas as required for purposes of poising the potential. A mixture of air with 2·5 % carbon dioxide was passed through the control vessel. With this amount of carbon dioxide the p_H of the media remained at about 7·4.

Both gas mixtures were passed through wash-bottles containing 0·9 % NaCl, which were kept at body temperature in the incubator. The gas was thus saturated with water vapour, and all danger of evaporation of the media was avoided. An open side-tube, at a point just before the gas entered the experimental vessel, dipped into water and served as an escape valve. The pressure of gas passing through the culture vessel could be nicely adjusted by varying the depth of the water. Rubber connections were reduced to a minimum and made where necessary with short pieces of pressure tubing.

Electrical measurements.

The E_h of the culture medium was measured by determining the potential set up on metal electrodes immersed in the solution. The circuit was completed by a half-cell formed by a bright platinum electrode immersed in $N/10$ hydrochloric acid saturated with quinhydrone; the half-cell was connected to the culture vessel by a saturated KCl-agar bridge. The quinhydrone $N/10$ HCl half-cell was used because it is easy to prepare and it will give a potential which is reproducible with considerable accuracy [Morgan *et al.*, 1931]. The potential of this half-cell was taken to be +623 mv. [Clark, 1928, p. 420]. The use of a saturated KCl-agar bridge introduced unavoidable errors of 2–3 mv. [Maclagan, 1929], but this is not serious for experiments involving differences of the order of 10 mv. The metal electrodes were carefully cleaned with hot chromic acid before each experiment by the method described by Morgan *et al.* [1931]. This was very necessary to ensure consistent behaviour of the electrodes in such poorly poised solutions as the media used. It is important to avoid taking any current from the metal electrodes, which are very easily polarised especially in poorly poised solutions. For this reason the use of a static electrometer is essential. The Lindemann electrometer proved to be very convenient and satisfactory. It was set up in the form used by Kerridge [1926] for measurements with the glass electrode. Two or more independent metal electrodes were used in each vessel. An individual electrode, apparently perfect, will sometimes be very inaccurate in these poorly poised solutions. It then has to be discarded.

In the early experiments both gold and platinum electrodes were used in each vessel. Dixon and Quastel [1923] have pointed out that in some sulphur-containing oxidation-reduction systems gold and platinum electrodes may behave differently. In our experiments the gold and the platinum electrodes behaved alike showing that the readings were not complicated by the presence of the anomalous systems described by Dixon and Quastel. In the later experiments only platinum electrodes were used. The electrodes were formed of thin platinum foil, 2 cm.^{2} in area, which were welded on to a short piece of platinum wire. The platinum wire was fused on to a piece of copper wire and was then fused into a glass holder with sealing-in glass.

In the early experiments a glass electrode (Haber's bulb form) was also immersed in the culture medium. The bulb of the electrode was blown from the special glass recommended by MacInnes and Dole [1930]. It was obtained from the Corning Glass Co., New York (No. 015). The glass electrode contained $N/10$ HCl and was fitted with a $N/10$ HCl quinhydrone half-cell. Special precautions were taken to ensure perfect insulation. By this means it was possible to determine whether there was any gross change of reaction of the culture medium during the experiment. If bacterial infection developed the medium turned rapidly acid (at the same time the E_h would be rapidly lowered), but in an uninfected culture the p_H remained practically constant for the duration of the experiment. Therefore, for the sake of simplicity, the glass electrode was discarded in the later experiments. The p_H of the medium was determined at the end of each experiment. In an uninfected experiment, in which the 2·5 % carbon dioxide mixtures were used, the p_H was always about 7·4. In an infected culture it had often fallen to below 7·0.

On passing the oxygen-free nitrogen and carbon dioxide mixture through the culture medium, the potential of the metal electrode, and therefore the E_h of the solution, fell more or less rapidly. If the unpurified nitrogen mixture was added to the gas stream as the required potential was approached the fall in E_h

was checked. By varying the rates of flow of the oxygen-free and the unpurified mixtures the E_h could be poised at the required level. The culture medium in this respect behaved like the media used by Knight and Fildes [1930].

Experimental procedure.

The cultures and electrode vessels, having been assembled, were placed in the incubator, and the electrical connections were made to the electrometer leads and to the standard quinhydrone half-cell. After allowing 30 mins. to 1 hour for temperature equilibration, the first potential measurements were made. The gas flow was then started; through the experimental vessel O_2-free nitrogen with 2·5 % CO_2; through the control vessel air with 2·5 % CO_2.

It was found that violent bubbling of gas through the fluid medium had for some unknown reason a deleterious effect upon the cultures. The reason for this is not clear, but it may be related to the fact that the growth-promoting power of embryo extract [Carrel, 1913] and of serum [Carrel and Ebeling, 1922] is said to be diminished by shaking. Whether this is an oxidation or merely a mechanical inactivation is not known. It was necessary, however, because of this phenomenon, to allow only a short spell of rapid bubbling to remove oxygen from the fluid and space above it, and then to cut down the rate of flow until the amount of agitation of the fluid was quite small. Throughout the experiment care was taken that the rates of flow of gas through experimental and control vessels were, as far as could be judged, the same. When the potentials registered by the electrodes in the experimental vessel had fallen almost to the desired level, unpurified N_2, *i.e.* N_2 containing a small quantity of oxygen, was introduced into the gas stream, and the rates of flow of purified and unpurified gas were adjusted so that the potentials came to and remained at this level. The experiment was continued for 40–48 hours, the gas flow in the experimental vessel being adjusted when necessary to maintain the constant potential. After 40–48 hours the coverslips were taken out of the vessels and the cultures, experimental and control, fixed in Bouin's fluid, stained with Mayer's haemalum, and examined. The p_H of the fluid medium was determined, at 38°, with the aid of a glass electrode of the Stadie type [Stadie *et al.*, 1931].

Results.

Fig. 2 shows the potentials which were observed in a typical experiment. Each curve relates to a single electrode, and in this experiment the gas flow was adjusted to maintain the E_h indicated by the electrodes in the experimental vessel at about 20 mv. It will be noted that the variation between the potentials of the three electrodes in the experimental vessel covers a range of about 20–30 mv., which is sufficiently small for the purposes of this work. In the control vessels the agreement is not quite so good. This might be expected where a considerable amount of oxygen is present. During the first 24 hours of the experiment there is a gradual drift of the potential to a more positive value. These characteristics were observed so consistently in all the experiments that in many of the later ones it was not considered necessary to make potential measurements in the control vessel.

Experiments were discarded in any of the following circumstances. (1) When there was marked disagreement between the behaviour of the electrodes in the same vessel. (2) When the E_h during the course of the experiment showed wide fluctuations owing to imperfect adjustment of the gas stream. (3) When the culture medium showed evidence of bacterial infection. (4) If for any unknown reason the control cultures did not grow well.

The following experiments we consider to be free from these defects: two in which the E_h was maintained at +120 mv., one in which it was maintained at +20 mv., one at −30 mv., and two at −100 mv. An indication of the activity of the cultures may be obtained by reference to Table I. To obtain the

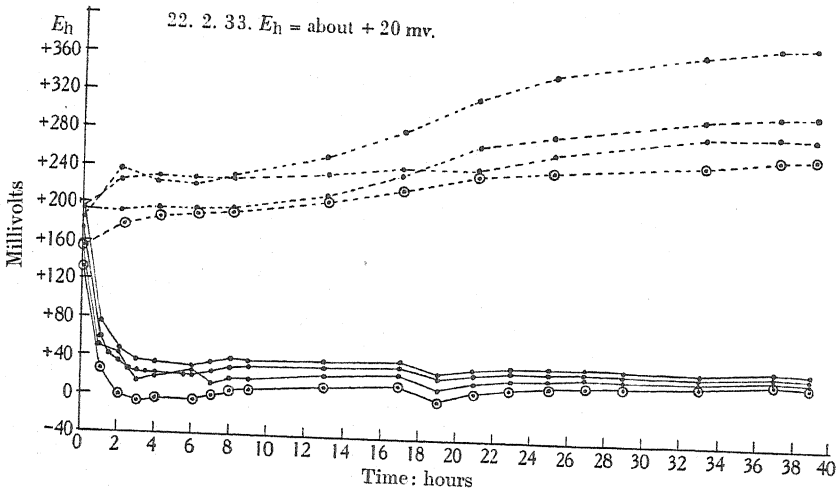


Fig. 2. Continuous line, experimental vessel. Broken line, control vessel. •—•, Platinum electrodes. ⊙—⊙, Gold electrodes.

Table I.

Areas in mm.²

No. of exp.	E_h of medium (mv.)		Area of explant A_1	Area of whole culture A_2	Area of outgrowth $A_2 - A_1$	Mean area of outgrowth of exp. culture as % of that of controls
1	+120	Exp.	0·27	1·87	1·60	
			0·34	2·38	2·04	34
		Contr.	0·42	5·57	5·15	
			0·37	5·85	5·48	
2	+120	Exp.	0·58	2·63	2·05	
			0·67	2·73	2·06	
			0·57	2·23	1·66	43
		Contr.	0·33	4·78	4·45	
			0·57	5·04	4·47	
3	+ 20	Exp.	0·44	1·07	0·63	
			0·45	1·91	1·33	12
		Contr.	0·54	7·62	7·08	
4	− 30	Exp.	0·64	1·06	0·42	
			0·69	0·81	0·12	2·3
		Contr.	2·34	15·25	12·91	
			1·95	13·05	11·10	
5	−100	Exp.	Only a few cells have migrated out			
		Contr.	0·93	5·93	5·00	
			0·97	4·72	3·75	
			0·86	4·96	4·10	
			0·58	3·38	2·80	
6	−100	Exp.	Only a few cells have migrated out			
		Contr.	Cultures infected			

figures recorded in this table the fixed and stained cultures were placed under the microscope, and with the aid of a drawing eye-piece the outlines of the central fragment and of the whole culture were traced on to a sheet of paper. The areas outlined on the paper were then measured with a planimeter, and knowing the magnification, the actual areas of the cultures were calculated. These are given in the table in mm.^{2} For the purpose of comparing the growth of experimental and control cultures the area of the outgrowth has been used. This is given by the difference between the area of the whole culture, A_2, and that of the central fragment, A_1. It was thought that this would be a truer representation of the activity than the fraction A_2/A_1 which is often used. A_1 in the latter expression ought really to be the original area of the explant, but the area of the explant when measured at the end of the experiment is sometimes, especially in well-growing cultures, considerably greater than its initial value. This is very noticeable in Exp. 4 in the table. When the difference A_2-A_1 is considered, however, the error due to this uncertainty about the true original area is much diminished, because A_1 is small compared with A_2. The final column of the table shows the average outgrowth of the experimental cultures expressed as a percentage of that of the control. It should be pointed out that in addition to diminution in the area of outgrowth in the series, there is at the same time a very marked decrease in the density of cell population in the outgrowth. This factor cannot conveniently be gauged numerically, but because of it the nett effect of reducing the E_h of the medium is really much more marked than the figures in the table suggest.

The apparent E_h in the control vessels was always of the order of +280 to +360 mv. When the E_h was maintained at +120 mv., the activity of the cultures was already markedly inhibited but was still considerable. In the two experiments the areas of outgrowth were 43 and 34 % respectively of those of the control cultures. Mitoses were still occurring at this E_h, as many as 19 being observed in a single culture. Examples can be seen in Plate VI, fig. 1. The appearance of the cells was not different from that of the control seen in Plate VI, fig. 2, though the number of mitoses was much smaller.

At an E_h of +20 mv. a much greater inhibition of activity was evident. The area of outgrowth was only 12 % of that of the controls and was more sparsely populated with cells. The condition of the cells was still fairly good, and there were one and four mitoses respectively in the two cultures (Plate VI, fig. 3). One or two of these appeared somewhat abnormal.

At −30 mv. the outgrowth was even smaller, only 2·3 % of that of the controls, but it cannot be said that activity was completely inhibited. There were no mitoses; the cells were heavily vacuolated and appeared to be on the verge of degeneration (Plate VI, fig. 4).

At −100 mv. only a few cells had migrated from the explant and it seems possible that this migration had occurred in the early stages, before the strongly reducing character of the environment had had time to overcome some innate capacity of the cells for resisting. Nevertheless the condition of the cells in the experimental cultures (Plate VI, fig. 5) appeared to be better than that shown in Plate VI, fig. 4. The reason for this is at present obscure.

The general result was therefore that when the potential indicated by the electrode was +20 mv. or more positive a considerable amount of activity was permitted. Even at a potential of +120 mv. there was a marked inhibition of growth, and this inhibition had reached a maximum at −30 mv. There was little difference, if any, between the amount of activity at −30 mv. and that at −100 mv. Even at these low levels some of the cells retained a limited power of

migration for a few hours at any rate, and did not appear to be killed within the time of the experiment.

Inhibition of mitosis appears to be complete at a potential which is rather more positive than that necessary for maximum inhibition of migration. Mitosis has almost completely ceased at +20 mv., and was not observed at −30 mv.

In some of the experiments at potential levels below +120 mv., the cells of the experimental cultures showed heavy vacuolation and distortion of nuclei suggesting imminent degeneration. These appearances were not however sufficiently regular to be related to the potential level. They must at present be ascribed to some unknown and as yet uncontrolled variable in the environment of the cultures.

It has been mentioned that Laser [1933] has recently stated that chick embryo tissue can grow equally well under aerobic or anaerobic conditions, but only when it has first been allowed to grow under normal conditions for 24 hours. Two experiments have been made to test this. The experiments were carried out in the same way as those described above, but the vessels with the cultures were left in the incubator undisturbed for 1–2 days before the flow of nitrogen was commenced. The experimental cultures were then allowed to grow for a further similar period of time at E_h+50 mv. The two experiments produced identical results. Fixing and staining of the cultures showed that the growth of the experimental cultures had been markedly inhibited, and the cells were much more heavily vacuolated than were the controls. There were several mitoses, but far fewer than in the controls. In many the mitotic figure was distorted in a striking fashion by the large vacuoles (probably fat) in the cytoplasm (Plate VI, fig. 6). In spite of the preliminary growth under aerobic conditions therefore the effects observed fit very well into the series of experiments already described and correspond with what was expected at that level of potential. Thus these results do not support Laser's conclusions.

Discussion.

In the experiments described above mitosis was observed when the oxygen pressure must have been considerably lower than Wright's minimum figure for the same type of tissue, *i.e.* 12 mm. Hg. This follows from the fact that the oxygen pressure in the unpurified nitrogen was only about 2 mm. Hg, and this was diluted by oxygen-free nitrogen before reaching the cultures. Mitosis therefore occurred when the oxygen pressure was much below 2 mm. Hg.

In considering these experiments, it is important to realise that the oxidation-reduction systems which determine the electrode potentials cannot be in reversible equilibrium with the oxygen of the gas phase. The arguments which apply to the experiments of Knight and Fildes were supported by our experience. An E_h of +100 mv. corresponds thermodynamically to an oxygen pressure of about 10^{-45} atmospheres. To maintain this E_h it was necessary to pass in a certain proportion of unpurified nitrogen containing about 0·2 vol. % of oxygen. Although the final composition of the gas phase was not determined it must, of course, have been far in excess of 10^{-45} atmospheres.

These considerations, together with the fact that the electrode potential can be raised or lowered by corresponding changes in the oxygen content of the solution, suggest that we may be dealing with a complex system somewhat as follows. We suggest, firstly, that the medium contains several reversible oxidation-reduction systems of an unknown nature. Each system is able to poise the potential, in the range of E_h about its own E_0 value, to an extent dependent upon the amount of the particular system present. The range of E_h covered by

all the systems together is such that some degree of poising can be manifested at any potential between, at any rate, +200 mv. and −100 mv. Secondly, the medium contains a reducing substance, or substances, which in the absence of oxygen can reduce the oxidised component of any of the reversible systems which determine the electrode potentials. In the absence of oxygen therefore this reduction takes place and the potential falls to a very low figure. Thirdly, in the experiments described, the medium contains oxygen. This, while not itself establishing equilibrium with the electrodes, tends to oxidise those systems which are in such equilibrium and therefore to raise the potential. A combination of these three factors might be expected to produce the observed results. The potential is set up by a reversible oxidation-reduction system effective at that point in the E_h scale. The condition of the reversible system is determined by the balance between oxidation of the system by oxygen and reduction of the system by a more strongly reducing substance in the medium.

The vigour of the reducing substances appears to vary in different samples of serum and embryo extract. In one experiment the E_h fell rapidly in the absence of oxygen to below −400 mv. In another experiment in which the same precautions were taken to eliminate traces of oxygen, the E_h did not fall below −20 mv. These results suggest that perhaps other factors besides oxygen pressure should be taken into account when considering the growth of tissues in low concentrations of oxygen. It is possible that the growth may be affected by the E_h more than by the oxygen pressure. The question, which of these two is the more important, cannot yet be regarded as settled. The possibility still remains that, whatever may be the factors governing the E_h, the growth of tissue is after all determined directly by the concentration of oxygen. More work upon the growth of cells under limiting conditions of oxygen pressure and of E_h is needed before this point can be decided.

It is of interest to consider these results in relation with those obtained by Needham and Needham [1925] and by Cohen *et al.* [1928], who studied the E_h of the protoplasm within amoebae by the micro-injection of oxidation-reduction dyes. From the results given by Needham and Needham the E_h within an amoeba appears to be maintained at about +100 mv. The experiments recorded above show that embryonic tissue cells, at any rate, can not only migrate but show mitosis when the medium surrounding them has E_h as low as +20 mv. It would be surprising if cells were able to live and multiply in a medium with E_h 80 mv. more negative than that within the cell itself. Our results are more in accord with those of Cohen *et al.*, which suggest that the E_h of the interior of the cell is below −130 mv. With E_h −100 mv. we find that embryonic cells of the chick are still able to survive to some extent, though there is no mitosis and little or no migration.

Summary.

1. On passing pure nitrogen through a culture medium containing serum and embryo extract the E_h of the medium fell rapidly.

2. By adding different small amounts of oxygen the E_h could be maintained at any desired level between +300 mv. and −100 mv.

3. The growth and division of cells from embryonic chick heart tissue have been studied when the E_h of the medium has been maintained at different levels. In general the lower the E_h the poorer has been the growth of the tissue culture.

4. All mitosis ceased between E_h + 20 mv. and −30 mv. Migration of cells had almost entirely ceased at an E_h of −30 mv.

5. The possible significance of these results is discussed.

REFERENCES.

Carrel (1913). *J. Exp. Med.* **17**, 14.
—— and Ebeling (1922). *J. Exp. Med.* **36**, 399.
Clark (1928). The determination of hydrogen ions. (Baillière, Tindall and Cox.)
Cohen, Chambers and Reznikoff (1928). *J. Gen. Physiol.* **11**, 585.
Dixon and Quastel (1923). *J. Chem. Soc.* **123**, 2943.
Fischer (1928). *Z. Krebsforsch.* **26**, 463.
Kerridge (1926). *J. Sci. Instr.* **11**, 404.
Knight and Fildes (1930). *Biochem. J.* **24**, 1496.
Laser (1933). *Biochem. Z.* **264**, 72.
MacInnes and Dole (1930). *J. Amer. Chem. Soc.* **52**, 29.
Maclagan (1929). *Biochem. J.* **23**, 309.
Michaelis (1930). Oxidation-reduction potentials. (J. B. Lippincott and Co., Philadelphia.)
Morgan, Lammert and Campbell (1931). *J. Amer. Chem. Soc.* **53**, 454.
Needham and Needham (1925). *Proc. Roy. Soc. Lond.* B **98**, 259.
Pannett and Compton (1924). *Lancet*, i, 381.
Stadie, O'Brien and Lang (1931). *J. Biol. Chem.* **91**, 243.
Warburg, Posener and Negelein (1924). *Biochem. Z.* **152**, 309.
—— and Christian (1931). *Biochem. Z.* **242**, 170.
Wind (1926). *Biochem. Z.* **179**, 384.
Wright (1928). *J. Path. Bact.* **31**, 735.

DESCRIPTION OF PLATE VI.

Fig. 1. Part of culture in medium at $E_h = 120$ mv., showing mitosis. ×400.

Fig. 2. Part of control to Fig. 1. ×400.

Fig. 3. Culture grown with medium at $E_h = 20$ mv., showing mitosis. ×400.

Fig. 4. Culture grown with medium at $E_h = -30$ mv. ×400.

Fig. 5. Culture grown with medium at $E_h = -100$ mv. ×400.

Fig. 6. Culture grown in air for 36 hours, then at $E_h = 50$ mv. for 48 hours. ×400.

Note distorted mitotic figure in the centre of the field. The equatorial plate of chromosomes is pushed towards one end of the cell and is no longer lying at right angles to the long axis of the cell, as the result of the presence of large vacuoles, probably representing fat.

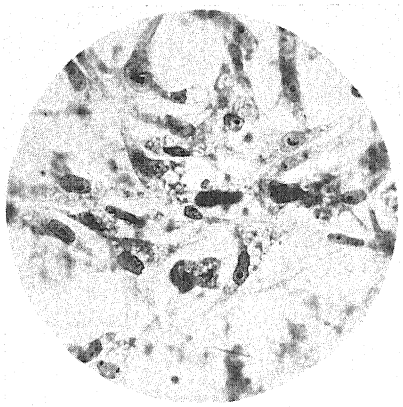
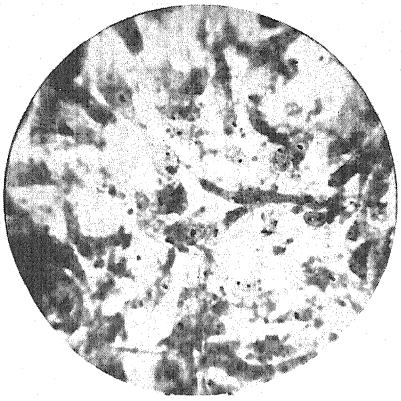


Fig. 1.



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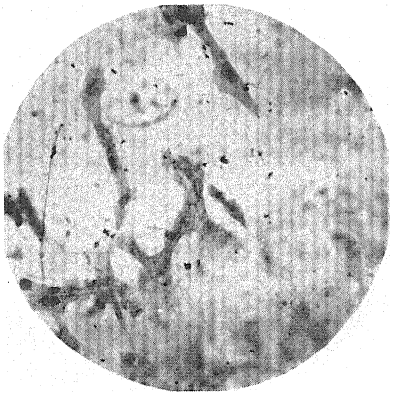

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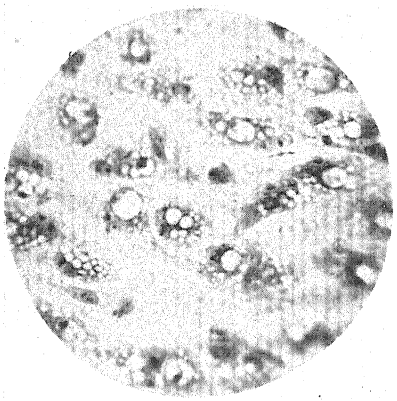

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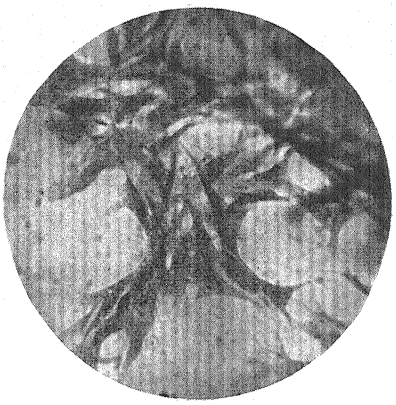

Fig. 5.

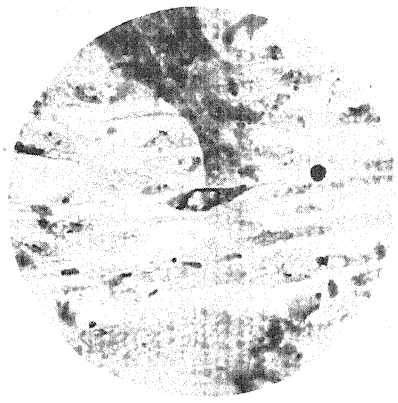

Fig. 6.

CLI. THE PRESENCE OF A SAPOTOXIN IN *XANTHOSOMA ATROVIRENS*, A TROPICAL FOOD-TUBER.

By ALFRED CLARK and ROY BASIL WATERS.

(*Received March 24th, 1934.*)

It has long been known that certain plants containing glucosides of the saponin group cause much disease and high mortality amongst domestic animals in different parts of the world [Ewart, 1931]. It has not however been shown previously that this class of poisons is present in harmful quantities in any plant used in human consumption, excepting perhaps the glyco-alkaloid in the potato (*Solanum tuberosum*) and the sapotoxin of *Agrostemma githago* seeds which are sometimes accidentally present in wheat and ground with it in milling.

The tubers of several plants of the natural order Araceae are largely consumed in tropical countries, and an account of the extraction from the one most extensively eaten in the West Indies of an acid sapotoxin which produces nephritis is of interest, especially in view of the heavy incidence of this disease amongst the poorest class of the population in Trinidad and other tropical countries.

The tuber selected for special examination is the Tannia (*Xanthosoma atrovirens*) as this is eaten all the year round whilst most of the others have their season. The wide adoption of tannia as a food is explained by the fact that it is palatable, abundant and cheap. Further, apart from its unfortunate toxicity, it has a fairly satisfactory composition as a foodstuff. During an investigation of its dietetic value it was found that the addition of cholesterol or of sterol-containing foods to a tannia diet materially lessened its toxic effects. This suggested the presence of a sapotoxin and attempts were made to isolate such a substance.

The peeled tubers were finely minced and mixed with distilled water. After several hours an equal volume of alcohol was added and the precipitated starch removed by filtration. The filtrate was freed from most of the alcohol by evaporation on the water-bath, and the hot solution was treated with 30 % normal lead acetate. A precipitate was formed and collected, but the filtrate retained some of its toxicity. Treatment with basic lead acetate yielded a further smaller precipitate and the filtrate from this, after removal of lead, was found to be free from toxic substance.

The normal lead acetate precipitate, after washing with distilled water, was suspended in water and treated with hydrogen sulphide. The resulting lead sulphide appeared to adsorb much of the toxic material which could not be separated entirely, either by prolonged treatment with hydrogen sulphide or by repeated boiling of the precipitate with water. The filtrate was evaporated under diminished pressure to a syrup from which an excess of cold alcohol precipitated a white hygroscopic solid. This solid had the chemical properties of an acid sapotoxin and showed a high toxicity when its solution in normal

saline was injected intravenously into rats. A large amount of inorganic impurities was present. Dissolution of the solid and repetition of the above processes removed most, but not all, of this impurity.

The alcoholic filtrate, on evaporation, yielded a dark gum. This was dissolved in water and treated with hot baryta solution. The precipitated barium salt was collected and well washed with water, and its suspension in water was treated with 10 % sulphuric acid in slight excess. The excess was neutralised with lead carbonate and the filtered solution evaporated under diminished pressure. The yellow residue was dissolved in alcohol and the solution decolorised with charcoal, filtered and evaporated. The clear gum thus obtained was dissolved in ethyl acetate and the solution filtered from inorganic material and evaporated. Repetition of the treatment with ethyl acetate gave a clear gum which crystallised on standing. Recrystallisation from ethyl acetate gave pure crystals of citric acid, M.P. 152°, either alone or when mixed with an authentic specimen. A solution of this product in normal saline produced little or no discomfort to rats when injected intravenously.

The basic lead acetate precipitate was similarly decomposed by hydrogen sulphide and the filtrate from the lead sulphide evaporated to a syrup. On pouring cold alcohol into this a cream-coloured solid was precipitated. This also proved to be a sapotoxin with an acid reaction and in physiological effect was indistinguishable from that obtained from the normal lead acetate precipitate. It was, however, much smaller in amount than the latter.

The alcoholic mother-liquors of this sapotoxin were evaporated to a gum, which was shown by its chemical and physiological properties to contain a sapotoxin. Various attempts were made to purify this gum, but without complete success. A solution of the material in water was mixed with freshly prepared magnesium hydroxide and evaporated to dryness at 50°, and the resulting mass was powdered and boiled with alcohol to remove any uncombined organic material. The solid was then suspended in water and a rapid stream of carbon dioxide passed through the mixture for several hours. The precipitated magnesium carbonate was removed by filtration and the solution evaporated to small bulk under diminished pressure and poured into a large excess of alcohol. The solid thus obtained was redissolved in a little water and again precipitated with alcohol. This gave a pale brown amorphous powder which still contained magnesium. Its solution in water was treated with basic lead acetate solution, the precipitate collected and well washed. This was decomposed by hydrogen sulphide and the filtrate evaporated to a syrup, which was dissolved in a little alcohol, filtered and diluted with a large volume of ether. This procedure gave a white, amorphous solid which still contained a small quantity of inorganic salts. It was extremely deliquescent, giving a brown gum on exposure to moist air for a few seconds, and possessed the properties of a sapotoxin.

The acid sapotoxins prepared in the above manner were believed to differ only in degree of purity. They were found to contain a little material which reduced Fehling's solution, and this was removed by boiling with alcohol. The product then contained 7 % of ash. Its solution was dextrorotatory and after hydrolysis with dilute sulphuric acid was less strongly dextrorotatory. The hydrolysed solution was neutralised with barium carbonate, evaporated to small volume under diminished pressure and poured into alcohol. A solid, presumably the sapogenin, separated out and was removed by filtration. The alcoholic solution was evaporated to dryness under diminished pressure. The residue reduced Fehling's solution and when heated with strong hydrochloric acid gave a very weak furfuraldehyde reaction to aniline acetate paper. An osazone was

obtained which, microscopically, resembled glucosazone, M.P. 194–196°. Owing to lack of material it was not possible to recrystallise the osazone. When, however, it was mixed with an authentic specimen of glucosazone, the M.P. was raised to 199–200°. The sapotoxin thus appears to contain a hexose which yields glucosazone.

It would be difficult to make even a rough estimate of the toxin content of the tubers owing to the great loss by adsorption during the processes required for its isolation and because the content of the tubers, as indicated by monthly feeding experiments on rats, showed a great variation in the duration of life of animals fed on them. This duration was on an average 4 days in January: 22 days in March, April, May and June: 7 days in the remaining months.

The sapotoxins thus extracted from tannia tubers are highly toxic to rats. Intravenous injection of 0·1 mg. killed a 60 g. rat instantaneously from cardiac and respiratory paralysis: 0·05 mg. killed in 20 minutes. Injection of smaller quantities produced in 50 minutes a rise of blood-sugar ranging from 176 to 415 mg. per 100 ml. At the same time the hepatic glycogen was found to be greatly diminished, usually to the merest trace. The urine, when obtainable, was albuminous. A solution of 1 part of sapotoxin in 20,000 parts of normal saline haemolyses blood *in vitro*, with reduction of the liberated haemoglobin. Spectroscopic examination shows the presence of the bands characteristic of haematoporphyrin. The cholesterol-saponin compound is neither toxic when injected, nor haemolytic.

The raw tubers have a burning effect upon the tongue which is sometimes severe but usually not unpleasant. Rats eat them readily and their average daily consumption when given no other food is 0·35 g. per g. rat, which is slightly more than the amount consumed of the stock diet. In spite of the adequate food consumption the animals lose weight from the commencement of the experiment. Within 24 hours the faeces become putty-like and clay-coloured. The abdomen becomes greatly distended and the urine albuminous. A gradually increasing lethargy follows which becomes profound as death approaches. Adrenaline injected intravenously rouses the animal rather dramatically, but the stupor returns in a short time and a further injection has little or no effect. The blood-sugar in the terminal phase of the experiment is greatly diminished, amounts as low as 45 mg. per 100 ml. being observed, whilst at the same time it is found that the hepatic glycogen is almost or quite absent.

The *post mortem* signs are remarkably constant. The caecum is enormously distended and its walls much thinned. The average weight of this organ in 27 examinations of rats fed on raw tannia was 14·6 % of body weight (one 20·7 %) compared with a weight of 1·47 % in rats fed on normal diet. The stomach and intestines contain blood in almost every case. The liver, lungs and kidneys are usually very dark, the cortex and medulla in the last-named being hardly distinguishable by their colour. Special interest is attached to the condition of the adrenal glands in view of the disturbed carbohydrate metabolism in tannia poisoning. In the normal rat these are of a pale skin colour and, in the albino rat of 60–70 g. body weight, account for about 0·0275 % of body weight, but in rats of the same strain and weight which have been poisoned by tannia are about 0·0390 % and vary in colour from a dull pink to a purplish tint similar to that of the kidney. Microscopically the kidneys show the appearances characteristic of an acute toxic nephritis. The glomeruli and secretory tubules are much disintegrated and the picture presented closely resembles that produced in poisoning by uranium nitrate. Granules of blood pigment are found abundantly scattered in kidneys, suprarenals and spleen and are especially plentiful in the

reticulo-endothelial cells of the liver. These appearances are found whether the tubers have been ingested or the toxin injected, but in the latter case the effects are more marked in the kidneys and less in the other organs than in the former.

Unstained blood films prepared in the ordinary way from rats poisoned by tannia or injected with its toxin show colourless crystals which are quite new in our experience. These are usually regular, stellate structures and many are six-pointed. They are about twice as large as a red blood corpuscle. We have not been able to find them in the films when freshly spread, but they can be discovered an hour later. They are not very numerous, but are striking and characteristic when found. It is not yet known whether they occur in cases of tannia poisoning in human beings in tropical countries. Ordinary haemoglobin crystals are sometimes found in association with the stellate crystals, being produced no doubt by the saponin haemolysis.

Summary.

A highly poisonous acid sapotoxin, for which the name tanniatoxin is suggested, has been isolated from the tubers of *Xanthosoma atrovirens*, a food commonly eaten in Trinidad under the name of "tannia."

When injected in doses of 0·1 mg. it kills a 60 g. rat instantaneously from paralysis of heart and respiration.

In smaller doses or when the raw tubers are eaten, a glomerulo-tubular nephritis is produced.

The sapotoxin haemolyses blood *in vitro* and converts the liberated haemoglobin into haematoporphyrin.

Certain stellate crystals which are found in blood films of poisoned rats are described.

It is suggested that certain cases of nephritis occurring in tropical countries may be caused by the consumption of tannia and other aroid tubers.

We desire to thank the London School of Hygiene and Tropical Medicine for their generous provision of laboratory facilities. We are also indebted to the Medical Research Council for whole time grants which have made the investigations possible.

REFERENCE.

Ewart (1931). *Council for Sci. and Ind. Res., Commonwealth of Australia, Melbourne,* Bull. 50.

CLII. THE EFFECT OF THYREOTROPIC HORMONE AND OF DESICCATED THYROID UPON CREATINE AND CREATININE EXCRETION.

By LEONARD IRVING PUGSLEY, EVELYN M. ANDERSON, and JAMES BERTRAM COLLIP.

From the Department of Biochemistry, McGill University, Montreal.

(*Received April 13th, 1934.*)

Cramer and Krause [1913] observed a creatinuria in both men and dogs as a result of feeding thyroid tissue. Gross and Steenbock [1921] found that feeding sheep thyroid to a pig on a nitrogen-free diet led to an increase in the creatine excretion. Patients with Graves' disease excrete excessive amounts of creatine in the urine. It has been suggested by Shorr *et al.* [1933] that the thyroid hormone is involved in the breakdown and building up of phosphocreatine. Eimer [1931] reported a significant increase in the total creatinine excretion of healthy subjects when fed desiccated thyroid. Recently, Shrire and Zwarenstein [1933] have shown that the administration of anterior pituitary extracts causes an increased excretion of creatinine in normal rabbits but not in castrated animals.

Methods.

Adult rats were placed on the following diet 3 weeks before the metabolism studies were started: wheat flour 1000 g., corn meal 1000 g., oatmeal 1000 g., linseed meal 300 g., wheat germ 82 g., brewer's yeast 182 g., cod-liver oil 180 ml. We have found the diet to be an important factor in studying creatine metabolism. The addition of meat or fish meal to the diet gives relatively high and irregular values for the creatine excretion. The rats were kept in Hopkins's metabolism cages and the urine and faeces collected separately. The urine was collected over a period of 48 hours, and the samples were made up to a volume of 20 ml.; the creatinine was estimated on a convenient aliquot by the method of Folin [1914].

The creatine was calculated by difference after autoclaving the urine in the presence of picric acid, and it is expressed here in terms of creatinine. The oxygen consumption was measured in a multiple chamber respiration apparatus, similar to that described by Benedict [1930–31]. The metabolic rate is expressed as the percentage increase in oxygen consumption over that determined in the pre-experimental period. Since fasting alone has an effect upon the creatine and creatinine excretion, the metabolic rates could not be taken under fasting conditions; however, on a series of several hundred rats we have found that the oxygen consumption, measured under feeding conditions at approximately the same hour each day, tends to be remarkably constant (unpublished data). Hypophysectomy was performed on the rats by the parapharyngeal approach of Selye [Collip *et al.*, 1933]. Two weeks were allowed after the operation before the creatinine studies were started.

The thyreotropic extract which was used showed on assay 100 units per ml. [Anderson and Collip, 1933]. One unit represents the smallest amount of the hormone which, when given twice daily to an hypophysectomised rat, will produce a 20 % increase in oxygen consumption within 96 hours.

Results.

Fig. 1 shows the effect of feeding desiccated thyroid upon the metabolic rate and the creatine and creatinine excretion of normal rats. The values in this chart represent the average of 6 rats. After a control period of 6 days, the rats were each given 2 grains of desiccated thyroid daily in their food. A very striking increase in creatine output occurred, which reached a peak on the twelfth day of thyroid feeding. It then commenced to drop off gradually, in

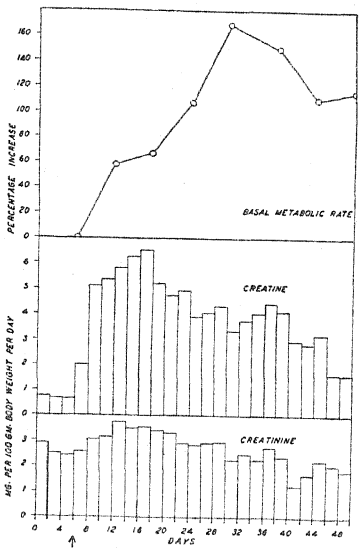

Fig. 1. The effect of feeding 2 grains of desiccated thyroid daily upon the metabolic rate and the creatine and creatinine excretion of adult rats.

spite of a rising metabolic rate. The creatinine excretion was increased slightly during the first 2 weeks of thyroid administration. After 24 days of thyroid feeding the animals were in a poor condition due to the extreme hyperthyroidism, and 3 rats of the series died before the experiment was finished. The drop in the metabolic rate which occurred in the sick rats lowered the average rate for the group.

In another series of experiments, a group of 4 rats was given thyreotropic hormone. The effect upon the metabolic rate and the creatine and creatinine excretion is shown in Fig. 2. Fifty units of thyreotropic hormone were injected intraperitoneally twice daily for a period of 32 days. A rise in the creatine excretion occurred immediately after beginning the injections, and the excretion then gradually fell to the normal level. The metabolic rate rose to a level of + 35 %, and then gradually fell, finally below the pre-experimental level.

Fig. 3 shows the effect of the thyreotropic hormone upon the metabolic rate and the creatine and creatinine excretion of hypophysectomised rats. A group

of 4 animals was studied. The thyreotropic hormone in doses of 50 units twice daily was started on the tenth day. It is seen that the creatine excretion reached a peak by the fourth day of injection and then began to decline while the metabolic rate was still rising. This suggests that the relationship between the two is not as direct as one might conclude from Fig. 2. The amount of creatinine

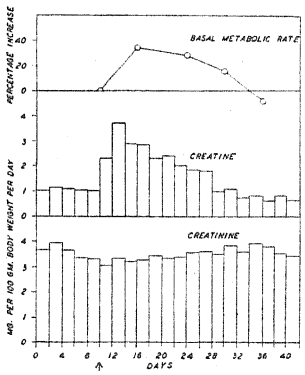

Fig. 2.

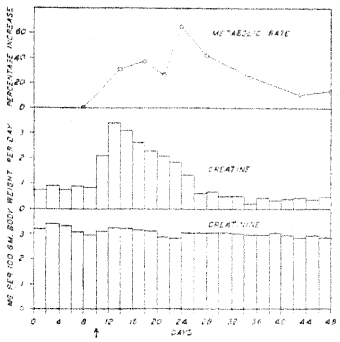

Fig. 3.

Fig. 2. The effect of 50 units of thyreotropic hormone upon the metabolic rate and the creatine and creatinine excretion of adult male rats.

Fig. 3. The effect of 50 units of thyreotropic hormone upon the metabolic rate and the creatine and creatinine excretion of hypophysectomised rats.

excreted remained unchanged throughout the experiment and was essentially the same as that of normal rats. This differs from the findings of Braier [1931], who reported a 35–40 % decrease in the creatinine excretion of hypophysectomised dogs.

The thyreotropic hormone produced a rise in the creatine output of the dog. This is shown in Table I. It will be noted that a diuresis occurred simultaneously with the creatinuria.

Table I. *Creatine and creatinine excretion of a dog treated with thyreotropic hormone. Wt. 26·6 kg.*

	Day	Urine volume ml. per day	Creatinine g. per day	Creatine as creatinine g. per day
Control period	2	867	1·103	0·357
	4	725	0·668	0·195
	6	1135	1·003	0·334
Experimental period. Injections started on 6th day; 500 units thyreotropic hormone twice daily, intravenously	8	1250	0·817	0·807
	10	1335	0·861	0·795
	12	1675	0·885	1·273
	14	1070	0·718	0·968
	16	1375	0·891	0·663
	18	1425	0·789	0·479
	20	1550	0·790	0·603
	22	1375	0·715	0·524
Recovery period. Injections stopped on 22nd day	24	1360	0·743	0·262
	26	1050	0·550	0·088
	28	930	0·756	0·055
	30	1000	0·515	0·110

The effect of small doses of thyreotropic hormone on creatine excretion has been studied. In Table II it is seen that 6 units of thyreotropic hormone are

Table II. *The effect of graded doses of thyreotropic hormone upon the creatine and creatinine excretion of normal rats.*

		Group I (average of 4 rats)			Group II (average of 4 rats)			Group III (average of 4 rats)		
	Day	Creatinine mg.	Creatine mg.	Metabolic rate	Creatinine mg.	Creatine mg.	Metabolic rate	Creatinine mg.	Creatine mg.	Metabolic rate
Control period	2	3·65	1·16	—	2·80	0·58	—	3·08	0·69	—
	4	3·33	1·09	—	3·22	0·64	—	2·96	0·36	—
	6	3·28	1·04	Normal	2·84	0·79	Normal	2·74	0·56	Normal
Injections started on 6th day		Dose: 50 units b.i.d.			Dose: 12 units b.i.d.			Dose: 6 units b.i.d.		
Experimental period	8	3·03	2·17	—	2·94	0·62	—	2·64	0·50	—
	10	3·31	3·71	—	2·81	0·74	—	2·64	0·57	—
	12	3·24	2·78	+34·8 %	2·82	1·20	—	2·73	1·06	—
	14	3·44	2·30	—	3·08	1·20	+17·3 %	2·93	1·27	+28·7 %
	16	3·32	2·41	—	3·03	0·53	—	3·15	0·28	—
	18	3·40	2·06	+28·1 %	3·09	0·44	+16·7 %	3·17	0·34	+19·1 %

The thyreotropic hormone in doses as low as 6 units twice a day causes an increase in creatine excretion in the normal rat. The creatine and creatinine are expressed in mg. per 100 g. body weight per day. The creatine is expressed in terms of creatinine. The "metabolic rate" is the percentage increase in oxygen consumption over that determined in the control period.

sufficient to cause a significant rise in the creatine excretion and metabolic rate of normal rats. In the hypophysectomised rat, 1 unit causes a more striking increase in the creatine output than is produced after 6 units in the normal rat (Table III).

Table III. *The effect of thyreotropic hormone upon creatine and creatinine excretion of hypophysectomised rats.*

		Group I (average of 4 rats)			Group II (average of 2 rats)			Group III (average of 2 rats)		
	Day	Creatinine mg.	Creatine mg.	Metabolic rate	Creatinine mg.	Creatine mg.	Metabolic rate	Creatinine mg.	Creatine mg.	Metabolic rate
Control period	2	3·34	0·77	—	2·85	0·77	—	2·65	0·95	—
	4	3·02	0·90	—	2·79	0·17	—	3·14	0·28	—
	6	2·90	0·83	Normal*	2·81	0·15	Normal*	2·77	0·18	Normal*
Injections started on 6th day		Dose: 50 units b.i.d.			Dose: 2 units b.i.d.			Dose: 1 unit b.i.d.		
Experimental period	8	3·10	2·12	—	2·95	0·14	—	3·00	0·12	—
	10	3·28	3·38	+31·5 %	2·94	2·42	+26·4 %	2·74	2·52	+20·9 %
	12	3·24	3·08	—	2·56	2·24	—	3·08	2·92	—
	14	3·18	2·62	+37·8 %	2·95	1·26	+11·1 %	2·90	2·62	+ 5·0 %
	16	3·14	2·30	—	2·82	0·80	—	2·83	1·07	—
	18	2·90	2·09	+26·3 %	2·78	0·10	+21·0 %	2·74	0·26	+10·9 %

The hypophysectomised rat is exceedingly sensitive to the thyreotropic hormone. It is seen here that one unit of the hormone, twice a day causes a significant rise in the creatine excretion.

* Normal for hypophysectomised rats.

Since fairly small amounts of the thyreotropic hormone will produce a significant increase in creatine excretion in the normal rat, this may give us a satisfactory method of assaying the hormone. The simplicity of the test would

be a distinct advantage over our present method of measuring the increase in metabolic rate in the hypophysectomised rat.

Purified extracts of the growth hormone and of the adrenotropic hormone have been injected into normal rats and the creatine and creatinine excretion followed over a period of 18 days. The effect of thyreotropic extract which had been inactivated by boiling for 2 hours at p_H 5 was also studied. The average daily output of creatine on the eighth day of the growth extract was 0·71 mg. per 100 g., of the adrenotropic 0·55 mg., and of the boiled thyreotropic 0·38 mg., while the controls receiving active thyreotropic hormone excreted 2·88 mg. of creatine on the eighth day of injection.

A substance which inhibits the action of thyreotropic hormone in raising the metabolic rate has been reported by Collip and Anderson [1934; Anderson and Collip, 1934]. Creatine and creatinine excretion has been studied on a group of normal rats receiving the antithyreotropic extract, together with the thyreotropic hormone. The results are given in Table IV. A fall in metabolic rate

Table IV. *The effect of antithyreotropic and thyreotropic hormones upon the creatinine and creatine excretion of normal rats.*

		Group I (average of 4 rats)			Group II (average of 4 rats)		
	Day	Creatinine mg.	Creatine mg.	Metabolic rate	Creatinine mg.	Creatine mg.	Metabolic rate
Control period	2	2·81	0·72	—	2·92	0·60	Normal
	4	2·94	0·51	—	2·88	0·57	—
	6	3·00	0·82	—	2·86	0·50	—
	8	2·74	0·59	—	3·02	0·48	—
	10	2·86	0·68	Normal	2·90	0·60	Normal
Injections started on 10th day		Dose: thyreotropic 25 units b.i.d.			Dose: thyreotropic 25 units b.i.d.; antithyreotropic extract 1 ml. b.i.d.		
Experimental period	12	2·50	0·82	—	2·98	0·81	—
	14	2·95	1·05	+16·7 %	3·01	1·54	—
	16	2·90	1·22	—	3·12	1·21	−11·6 %
	18	3·10	1·74	—	3·14	1·15	—
	20	2·74	1·05	+37·4 %	2·58	1·07	− 6·0 %
	22	2·67	1·06	—	3·10	1·12	—
	24	2·70	0·82	—	3·00	0·89	—
	26	2·88	0·42	+20·1 %	3·18	0·66	−12·9 %
	28	2·66	0·76	—	2·88	0·51	—
	30	2·64	0·40	—	3·24	0·51	−20·1 %
	32	3·10	0·30	—	3·18	0·57	—
	34	2·80	0·20	+ 2·0 %	3·12	0·53	—

occurred in the group of animals receiving the antithyreotropic substance together with the thyreotropic hormone (Group II); however, the creatine excretion in this group was practically the same as in the group receiving thyreotropic hormone only (Group I).

Summary.

1. The feeding of desiccated thyroid to normal rats caused a rise in the creatine excretion, which reached its peak on the tenth day and then gradually decreased while the metabolic rate was still rising.

2. The thyreotropic hormone caused a temporary increase in creatine excretion both in the normal and the hypophysectomised rat.

3. A significant increase in creatine excretion was produced with 6 units of thyreotropic hormone in the normal rat and with one unit in the hypophysectomised rat.

4. The thyreotropic hormone caused an increase in the creatine excretion of the dog.

5. Purified extracts of the growth hormone and of the adrenotropic hormone had no effect on the creatine excretion of normal rats. Thyreotropic extract inactivated by boiling also gave negative results.

6. Antithyreotropic substance, which inhibited the rise in metabolic rate by thyreotropic hormone, did not prevent an increase in creatine excretion.

REFERENCES.

Anderson and Collip (1933). *Proc. Soc. Exp. Biol. Med.* **30**, 680.
—— —— (1934). *Lancet*, i, 784.
Benedict (1930–31). *J. Nutrition*, **3**, 161.
Braier (1931). *Compt. Rend. Soc. Biol.* **107**, 1195.
Collip and Anderson (1934). *Lancet*, i, 76.
—— Selye and Thomson (1933). *Arch. Path. Anat.* **290**, 23.
Cramer and Krause (1913). *Proc. Roy. Soc. Lond.* B **86**, 550.
Eimer (1931). *Z. ges. exp. Med.* **77**, 455.
Folin (1914). *J. Biol. Chem.* **17**, 469.
Gross and Steenbock (1921). *J. Biol. Chem.* **47**, 45.
Shrire and Zwarenstein (1933). *Biochem. J.* **27**, 1337.
Shorr, Richardson and Wolff (1933). *Proc. Soc. Exp. Biol. Med.* **31**, 207.

CLIII. THE INFLUENCE OF VITAMIN C (ASCORBIC ACID) ON PLANT AND ANIMAL AMYLASES.

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In a recent publication from this laboratory Purr [1933] reported on the effect of ascorbic acid on the action of the proteolytic enzymes. The present paper deals with the influence of ascorbic acid on plant and animal amylases which are important in cancer research on account of their relation to carbohydrate metabolism.

Kuhn [1925], in a fundamental work, studied the mutarotation of the products formed in the first stages of starch hydrolysis by amylases. He found that one group of amylases yields primarily α-maltose, which mutarotates downward (α-amylase), while another group yields β-maltose, which mutarotates upward (β-amylase). The amylases in pancreas and saliva belong to the α- type, while those of sprouted and unsprouted grains are of the β- type. These findings were later confirmed by Reichel [1932]. By studying the relation between the iodine reaction and the mutarotation of the starch decomposition products, it was found that the two types of amylase differ in their mode of action on the starch complex from the first point of contact. Thus, α-amylases are believed to break down first those groups in the starch complex which are responsible for the blue colour with iodine. Characteristically, the blue colour disappears with very low saccharification. On the other hand, β-amylases break down the starch in such a way that saccharification is almost complete while the blue colour remains unaltered. Through the discovery of this relation between saccharification and coloration with iodine, a simple method became available for distinguishing the amylases found in nature. It is merely necessary to allow the hydrolysis to proceed until a very definite colour tone, preferably the change from ultramarine blue to blue-violet, is reached and to determine the saccharification at that point. This is known as the transition point, and it expresses the amount of saccharification (in percentage maltose formed) at the point of colour change.

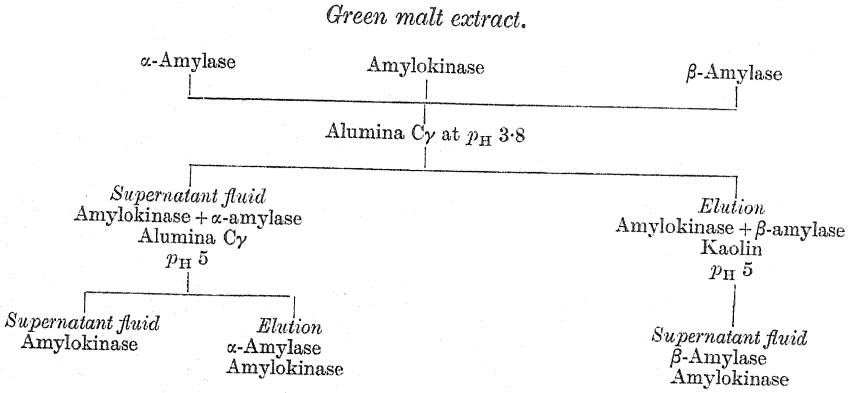
The transition points for several different types of amylase are as follows:

Transition point (percentage maltose formed).

Plant amylases		Animal amylases		
Barley	Green malt	Pancreas	Liver	Saliva
102 %	84 %	20 %	15 %	11 %
β-Amylase		α-Amylase		
Mutarotation upward		Mutarotation downward		
High saccharification		Low saccharification		
Iodine colour, blue-violet		Iodine colour, blue-violet		

By means of adsorption methods, Waldschmidt-Leitz, Reichel and Purr [1932] were able to isolate two amylases, α and β, from barley and green malt. While both amylases extracted from green malt were fully active, in barley the α-form was totally inactive and the β-form partially active. The difference between the barley enzyme system and the malt system lies in the formation of an activator of organic nature, "amylokinase," which arises during the process of sprouting.

The following scheme represents the process of separating the amylases of green malt.



This method, when applied to animal amylase systems, was unfortunately not so successful. However, the transition point of pancreas amylase indicates the presence of an animal β-type. As a result of the discovery, reported in this paper, of the specific activating effect of vitamin C on animal β-amylase, it has become possible to demonstrate the existence in pancreas of a β-type of amylase.

Vitamin C and animal amylases.

The effect of vitamin C on the activity of various animal amylases was investigated, using as source of enzyme the following: human saliva (0·2 ml. with 0·015 amylase units), acetone-ether-dried pig pancreas (0·1 ml. of a 1 : 10 aqueous extract with 0·035 amylase units), and rat-liver (1·5 ml. of a 1 : 5 aqueous extract with 0·0014 amylase units). To separate portions of the designated quantities of each of these were added (*a*) 8 mg. of ascorbic acid in 1 ml. of H_2O, (*b*) 8 mg. oxidised ascorbic acid in 1 ml. of H_2O, or (*c*) 1 ml. of H_2O. These mixtures were allowed to stand at 0° and p_H 7 for 5 minutes, after which 50 ml. of the stock solution were added. This contained 250 mg. of soluble starch (Lintner), 10 ml. of $M/15$ phosphate buffer of p_H 6·8 (or 10 ml. of $M/10$ citrate buffer of p_H 5·1), and 2·0 ml. of $M/5$ NaCl solution, in a total volume of 50 ml. The mixtures were incubated at 37° for the indicated periods of time, after which the enzyme action was stopped by addition of 2 ml. of N HCl. The amount of saccharification was determined by the method of Willstätter and Schudel [1918], the results being expressed in percentage maltose formed. The oxidised ascorbic acid was prepared by bubbling oxygen for 4 hours through a solution of 8 mg. of ascorbic acid in 1 ml. of H_2O at p_H 7. A trace of $FeSO_4,7H_2O$ was added to hasten the oxidation, the completion of which was determined by iodine titration.

Table I. *Ascorbic acid and animal amylases. Effect of* p_H.

Additions	p_H	Saliva		Pancreas		Liver	
		Time mins.	Maltose %	Time mins.	Maltose %	Time mins.	Maltose %
Water	6·8	10	22·6	8	37·04	60	13·72
Ascorbic acid	6·8	10	21·95	8	48·00	60	24·00
Oxidised ascorbic acid	6·8	10	22·00	8	46·64	—	—
Water	5·1	20	15·5	25	20·58	—	—
Ascorbic acid	5·1	20	15·9	25	19·20	—	—

From the figures obtained it can be seen that of the animal amylases tested, only the salivary amylase remained inactivated at both p_H 6·8 and 5·1. Pancreas and liver amylases, on the other hand, undergo considerable activation, but only at the optimum p_H for the enzyme reaction (6·8). In this respect the activation differs from that obtained with calcium salts, which occurs only at p_H 5·1, but not at 6·8.

The salivary amylase, as has been mentioned, has the lowest transition point found as yet, and therefore represents the purest α-type. The higher transition points of pancreas and liver amylases indicate the presence of mixtures of both α- and β-types. It was of interest to determine, therefore, whether these facts could be correlated with the non-activation of salivary amylase and the activation of pancreas and liver amylases by ascorbic acid. The investigations carried out in this direction, as shown in Table II, indicate that the activations obtained with ascorbic acid are related to a specific activation of the animal β-amylase.

Table II. *The effect of ascorbic acid on the transition point of pancreatic amylase.*

	Without ascorbic acid			With ascorbic acid			
	Saccharification				Saccharification		
Time mins.	Maltose mg.	Maltose %	Iodine colour	Iodine colour	Maltose mg.	Maltose %	
0	0·0	0·0	Ultramarine-blue	Ultramarine-blue	0·0	0·0	
4	20·0	8·0	,,	,,	37·5	15·0	
Transition point 10	45·0	18·0	Blue-violet	Blue-violet	70·0	28·0	Transition point
15	63·8	25·5	Violet	Violet	86·3	34·5	
20	80·0	32·0	Violet-red	Violet-red	102·5	41·0	
25	95·0	38·0	Red-violet	Red-violet	120·0	48·0	
30	112·5	45·0	Red-brown	Red-brown	136·3	54·5	

A large volume of a reaction mixture was prepared, containing 250 mg. of soluble starch, 10 ml. of $M/15$ phosphate buffer of p_H 6·8, 2 ml. of $M/5$ NaCl solution, and 1 ml. of a 1 : 10 aqueous extract of pancreas containing 0·015 amylase units (or the same amount of enzyme previously activated by 8 mg. of ascorbic acid), in a total volume of 50 ml. The mixture was incubated at 37°. The course of the reaction towards the transition point was followed by the method to be described later. At definite intervals, 50 ml. portions of the reaction mixture were removed and treated with 2 ml. of N HCl, and the amount of maltose formed was determined.

As shown in Table II, the activating effect of ascorbic acid is linked with an increase in the transition point. This indicates that ascorbic acid has a specific

activating effect on the β-amylase, and that the latter is present in pancreas and liver in an almost inactive state naturally.

Waldschmidt-Leitz and Purr [1932] were able to report an activating effect on animal amylase by calcium salts. As previously mentioned, this form of activation is sharply differentiated from that obtained with ascorbic acid in the conditions of p_H necessary. The same conclusion is reached by a study of the transition point, as shown in Table III.

Table III. *Activation of pancreatic amylase by $CaCl_2$ at p_H 5·1. Transition point.*

	Without $CaCl_2$				With $CaCl_2$			
	Saccharification				Saccharification			
	Time mins.	Maltose mg.	Maltose %	Iodine colour	Iodine colour	Maltose mg.	Maltose %	
	0	0·0	0·0	Ultramarine-blue	Ultramarine-blue	0·0	0·0	
	10	6·3	2·50	,,	,,	30·00	12·00	
	15	18·75	7·50	,,	Blue-violet	47·00	18·80	Transition point
	20	30·0	12·0	,,	Violet	62·50	25·00	
Transition point	25	47·5	19·0	Blue-violet	Violet-red	82·5	33·00	
	35	65·0	26·0	Violet	Red-violet	100·0	40·0	

Additions and procedure were the same as those for Table II, except that 10 ml. of $M/10$ citrate buffer of p_H 5·1 were used instead of the phosphate and 12 mg. of $CaCl_2$ instead of the ascorbic acid.

Calcium salts do not change the transition point, but only hasten its appearance, while ascorbic acid increases it. It is evident from these results that the effect of calcium salts is fundamentally different from that of ascorbic acid.

Procedure for the determination of the transition point.

Each of a series of test-tubes contained 2 ml. of distilled water and 3 drops of $N/100$ iodine solution. At definite time intervals, 1 ml. of the enzyme reaction mixture was added, until the first definite blue-violet colour could be recognised. Immediately, 50 ml. of the hydrolysing mixture were removed, 2 ml. of N HCl were added to stop the enzyme reaction, and the sugar formed was estimated by the Willstätter-Schudel method.

It should be mentioned that the method can be used for quantitative comparisons only when no substances are present which take up iodine in neutral or acid solution. When these conditions exist, the method is in all cases inapplicable; for the presence of large quantities of such reducing substances (cell residue, tissue suspensions, thiosulphate, *etc.*), leads to the formation of hydrogen iodide or its salts. The latter compounds, as is known, weaken the colour of the starch-iodine complex, and therefore interfere with the colour comparison. These substances affect the starch-iodine colour in a manner similar to that of amylase itself and may easily lead to erroneous conclusions.

In order to eliminate this source of error when using ascorbic acid, which takes up iodine very readily, it is necessary, in the determination of the transition point, to compensate for its presence. The method of procedure was the same as previously described, except that to those test-tubes in which the

transition point of pancreatic amylase alone was to be determined, 0·16 mg. of ascorbic acid was previously added. This amount corresponds to that present in 1 ml. of the enzyme mixture which is being activated by ascorbic acid. In each case, then, equivalent amounts of ascorbic acid were present, so that the error due to formation of hydrogen iodide was eliminated. Because of the consumption of iodine which occurs in the presence of ascorbic acid, it was necessary to add 10 instead of 3 drops of $N/100$ iodine to each test-tube, in order to have the necessary excess for the determination and for stopping the enzyme reaction.

The next series of experiments (Table IV) shows the effect of several amino-acids and of insulin on the activity of pancreatic amylase at p_H 6·8 and 5·1. The procedure was exactly the same as that described in Table I, except for the substitution of the indicated amounts of amino-acids and insulin for the ascorbic acid.

Table IV. *The effect of amino-acids on the activity of pancreatic amylase.*

	p_H 6·8			p_H 5·1		
Additions	Time mins.	Saccharification Maltose %	Activation Maltose %	Time mins.	Saccharification Maltose %	Activation Maltose %
None	7	14·7	0	20	12·00	0
Cysteine (20 mg.)	7	14·9	0	20	18·00	6·0
Cystine (20 mg.)	7	14·7	0	20	21·50	9·50
Alanine (20 mg.)	7	14·7	0	20	12·00	0
Histidine monohydrochloride (20 mg.)	7	14·8	0	20	23·00	11·0
Proline (20 mg.)	7	14·5	0	20	12·00	0
Insulin—132 units (9·5 mg.)	7	14·8	0	20	22·00	10·00

It is clear from this table that the p_H of the amino-acid activation agrees with that observed in the case of calcium activation. The agreement also can be seen in a study of the transition points (these figures are not presented in this paper). This activation therefore also differs sharply from that obtained with ascorbic acid.

The extent to which the activating influence of ascorbic acid is exerted on the complicated mechanism of glycolysis will be reported in a later communication.

Vitamin C and plant amylases.

The significance of ascorbic acid as an enzyme regulator is not limited to the amylases of animal origin, but as the following experiments show, it must also be credited with a similar function with regard to plant amylolytic processes.

The plant amylases investigated were the following: barley (0·5 ml. of a 1 : 5 aqueous extract, with 0·030 amylase unit), green malt (0·25 ml. of a 1 : 5 aqueous extract with 0·030 amylase unit), barley amylase purified by alumina Cγ, β-form (1·0 ml. with 0·015 amylase unit), and the green malt amylase purified by alumina Cγ, α-form (3·0 ml. with 0·015 amylase unit). To the designated amounts of each of these were added (*a*) 8 mg. of ascorbic acid in 1 ml. of H_2O, (*b*) 8 mg. of oxidised ascorbic acid in 1 ml. of H_2O or (*c*) 1 ml. of H_2O. The mixtures were allowed to stand 10 minutes at 37°, after which 50 ml. of the stock solution of p_H 5·1 previously described were added. The incubation and sugar determinations were carried out exactly as previously described. The purified β-amylase from barley was prepared by treating 10 ml. of 1 : 5 barley extract containing 1·5 amylase units with alumina Cγ (8 ml. = 60 mg. Al_2O_3) at

p_H 3·8 (2 ml. of *M* acetate buffer). The adsorbate was eluted with 10 ml. of *M*/15 secondary phosphate and brought to p_H 7 by the addition of *N*/10 HCl. The purified α-amylase was prepared by adsorbing 10 ml. of 1 : 5 green malt extract eight times with alumina Cγ (145 mg. × 8 of Al_2O_3) at p_H 3·8 (2 ml. of *M* acetate buffer). Compare the schema, p. 1142.

Table V. *Effect of ascorbic acid on plant amylases.*

Enzyme	No additions		Ascorbic acid		Oxidised ascorbic acid	
	Time mins.	Maltose %	Time mins.	Maltose %	Time mins.	Maltose %
Barley (unpurified)	9	35·68	7	12·34	7	36·36
Malt (unpurified)	10	37·04	10	19·90	10	28·81
β-Amylase (purified from barley)	20	37·70	20	10·37	10	36·36
α-Amylase (purified from malt)	9	19·90	10	16·46	10	11·90

While the animal amylases are either activated or else not affected at all by ascorbic acid, the plant amylases thus far investigated are inhibited. This inhibition is dependent not only on whether reduced or oxidised ascorbic acid is used, but also on the type of amylase, α or β. As shown in Table V, the β-type of plant amylase is very strongly inhibited by reduced ascorbic acid, but is unaffected by the oxidised form. This behaviour is shown both by the purified enzyme, and by the crude green malt extract which contains amylokinase. The opposite picture appears in the case of plant α-amylase, whose activity, as has been previously mentioned, is dependent on the presence of amylokinase. The activity of this form is inhibited only by the oxidised form of ascorbic acid, while it is scarcely affected by the reduced form. The slight inhibition which may be observed can be explained by the presence of a small amount of β-amylase which remains after the purification.

These findings extend the knowledge of the essential differences between the plant and animal amylases which are summarised in Table VI.

Table VI. *Comparative behaviour of plant and animal amylases toward various reagents.*

+ = Activation; − = Inhibition; 0 = Indifferent.

Reagent	p_H	Plant amylases		Animal amylases	
		β	α	β	α
Amylokinase	5·1	+	+	0	0
,,	6·8	+	+	0	0
Vitamin C	5·1	−	0	0	0
,,	6·8	−	0	+	0
Oxidised vitamin C	5·1	0	−	0	0
,,	6·8	0	−	+	0
NaCl	5·1	0	0	0	+
,,	6·8	0	0	0	+
$CaCl_2$	5·1	0	0	0	+
,,	6·8	0	0	0	0
p_H optimum		5·1	5·1	6·8	6·8

Table VII shows the effect of oxygen on the plant amylase complex from barley in the presence and absence of ascorbic acid. Oxygen was bubbled through a barley amylase extract for 4 hours in the presence and absence of ascorbic

acid. A trace of $FeSO_4$ was added as an oxidation catalyst. The determinations of amylase activity after the oxygenation were carried out exactly as previously described. The results obtained indicate that ascorbic acid may also exert an effect in protecting the amylase from oxidation.

Table VII. *Effect of oxygen on barley amylase in the presence and absence of ascorbic acid.*

	Barley amylase without vitamin Maltose (%)	Barley amylase with 8 mg. vitamin Maltose (%)
4 hour treatment with oxygen	12·3	20·6

Recently Virtanen *et al.* [1933] found a relation between growth of plants and vitamin C content. In view of the connection between vitamin C and the amylases reported in the present paper, it is of interest to present the results of an investigation[1] of the changes occurring in the activities of the several kinds of amylases during the ripening of various grains.

The crushed grains at different stages of ripening were extracted for 24 hours with 5 parts of water, toluene being added as antiseptic, and then filtered by suction. The reaction mixtures consisted of 140 mg. of amylo-amylase (prepared according to Samec), 10 ml. of citrate buffer of p_H 5·1, and aliquot parts of the grain extracts containing 0·01 amylase unit, in a total volume of 50 ml. The mixtures were incubated at 37°, and the course of the reaction towards the transition point was followed as previously described. The mutarotation was determined by the method of Kuhn [1925]. A 2 dm. polarimeter-tube was filled with the reaction mixture, and the rotatory power of the solution was determined at intervals, first without carbonate, and again after ½ hour standing with 0·5 g. of anhydrous sodium carbonate. The difference between the two determinations expresses the mutarotation, the magnitude of which is dependent on the experimental procedure. The decisive point is the direction of the mutarotation (− for α-; + for β-amylase).

Table VIII. *Relation between amylase activity and the ripening process of various grains.*

Grain	State of grain content	Transition point Maltose %	Mutarotation at transition point	Remarks
Barley	Milky fluid	77·5	+0·07°	For comparison, green malt has a mutarotation of +0·15 at the transition point (84 % maltose)
	Soft mass	67·3	+0·06	
	Mealy mass	82·1	+0·08	
	Ripe grain	96·1	+0·10	
Rye	Milky fluid	88·2	+0·13	
	Soft mass	68·0	+0·08	
	Mealy mass	76·0	+0·09	
	Ripe grain	83·3	+0·05	
Oats	Milky fluid	80·85	+0·15	
	Soft mass	60·7	+0·08	
	Mealy mass	75·0	+0·06	
	Ripe grain	90·0	+0·10	

On the basis of these results, it can be seen that in the first stages of the development of grains, the relationships between the different amylase types

[1] This investigation was carried out at the Biochemical Institute of the Deutsche Technische Hochschule, Prague. The various kinds of grains used in each stage of the ripening process were obtained from J. Purr, Grabschuetz, Czechoslovakia.

(α or β) are similar to those existing in sprouting grains such as green malt. The nearer the grain approaches to ripening, the higher is the transition point, until it finally reaches the value for fully ripened, dormant grain. The increase in the transition point indicates that the α-amylase is gradually becoming inactive, and from this it may be concluded that the α-enzyme in growing (including sprouting) plant cells is involved as an energy-supplying factor in the enzymic intermediate processes. It is probable that the decrease in vitamin C during the ripening of grains, as found by Virtanen *et al.* [1933], is related to the inactivation of the α-amylase. This working hypothesis is supported by the finding that oxidised vitamin C inhibits plant α-amylase. Whether amylokinase arises during the ripening process, and its relation to vitamin C, must be determined by further experiment.

SUMMARY.

1. It has been shown that vitamin C (ascorbic acid) is a specific activator for the β-type of animal amylase. β-Amylase occurs in animal organs in a practically inactive state.

2. Vitamin C exerts an inhibiting effect on the β-type of plant amylase but has no effect on the α-type. The oxidised form of ascorbic acid has no effect on the β-form but inhibits α-amylase of plants.

3. A study of the relation between amylase activity and the growth of grains leads to the conclusion that in the first stages of growth the same amylase relationships exist as in the sprouting grain. The fact that the α-components become inactive at the end of the growth period is in agreement with the findings of Virtanen on the vitamin C changes which occur, and with the fact that the oxidised form of the latter inhibits α-plant amylase.

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REFERENCES.

Kuhn (1925). *Liebig's Ann.* **443**, 1.
Purr (1933). *Biochem. J.* **27**, 1703.
Reichel (1932). Dissertation. Deutsche technische Hochschule, Prag.
Virtanen, Hausen and Saastamoinen (1933). *Biochem. Z.* **267**, 179.
Waldschmidt-Leitz and Purr (1932). *Z. physiol. Chem.* **213**, 63.
—— Reichel and Purr (1932). *Naturwiss.* **20**, 254.
Willstätter and Schudel (1918). *Ber. deutsch. chem. Ges.* **51**, 780.